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Asynchronous Synapse Elimination in Neonatal Motor Units: Studies Using GFP Transgenic Mice

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Summary

In developing muscle, synapse elimination reduces the number of motor axons that innervate each postsynaptic cell. This loss of connections is thought to be a consequence of axon branch trimming. However, branch retraction has not been observed directly, and many questions remain, such as: do all motor axons retract branches, are eliminated branches withdrawn synchronously, and are withdrawing branches localized to particular regions? To address these questions, we used transgenic mice that express fluorescent proteins in small subsets of motor axons, providing a unique opportunity to reconstruct complete axonal arbors and identify all the postsynaptic targets. We found that, during early postnatal development, each motor axon loses terminal branches, but retracting branches withdraw asynchronously and without obvious spatial bias, suggesting that local interactions at each neuromuscular junction regulate synapse elimination.

Introduction

One of the ways synaptic circuitry of the mammalian nervous system changes during development is the removal of axonal connections (Chen and Regehr, 2000; Lohof et al., 1996; Purves and Lichtman, 1980). To date, this process has been analyzed in greatest detail in the skeletal neuromuscular junction. In rodents, synapse elimination peaks in postnatal life, long after axons first contact muscle fibers. For example, in mice, outgrowing motor axons first form functional synaptic contacts roughly 1 week before birth. By birth, each muscle fiber possesses one synaptic site (the neuromuscular junction), but, almost invariably, more than one axon innervates that site (see Fladby and Jansen, 1988; Lichtman and Colman, 2000; Sanes and Lichtman, 1999). Over the first several postnatal weeks, multiple innervation of neuromuscular junctions decreases sharply. At each junction, the process of synapse elimination occurs gradually (Balice-Gordon and Lichtman, 1993), with the losing terminal branch retracting rather than degenerating. As synapse elimination proceeds, the junction remodels. At birth, intermingled branches of more than one axon overlie the acetylcholine receptors (AChRs) at the junctional site. Over the next several days or weeks, the branches of different axons gradually segregate, with one axon gaining area and strength as others lose contact area and efficacy (Balice-Gordon et al., 1993; Colman et al., 1997; Gan and Lichtman, 1998; Kopp et al., 2000). Generally, multiple innervation of rodent neuromuscular junctions is exceedingly rare after 2 weeks of age.

In most muscles studied to date, there is no change in the number of motor neurons innervating muscles during the postnatal stage when axonal inputs are removed (Brown et al., 1976; see, however, Bennett et al., 1983). Moreover, there is usually no change in the total number of muscle fibers during synapse elimination (Ontell and Kozeka, 1984a, 1984b; see, however, Betz et al., 1979). Given these facts, it has been argued that synapse elimination occurs as axons undergo a net reduction in the number of muscle fibers they innervate (i.e., a reduction in motor unit size). This idea is supported by the observation that the proportion of total muscle force generated by a single axon drops as development proceeds (Brown et al., 1976; for references, see Jansen and Fladby, 1990). Thus, synapse elimination is thought to be associated with a change in the branching of individual motor axons. No studies, however, have examined this phenomenon directly. Indeed, to date, the only method available in mammals to directly study muscle fiber distribution of single motor units has been the glycogen depletion technique (Edström and Kugelberg, 1968). In these experiments, a single motor neuron is stimulated until it exhausts muscle fiber glycogen stores, then, by staining sections of the muscle for glycogen, the stimulated fibers can be identified. Such studies have supported the idea that there is a net decrease in the size of neonatal motor units over time (Balice-Gordon and Thompson, 1988; Thompson et al., 1984). However, these experiments are technically difficult, especially in young muscles, making many of the conclusions open to debate (Jansen and Fladby, 1990). Moreover, glycogen depletion provides no information on the branching pattern of a motor axon.

Because of these technical limitations, it remains unclear how the branching pattern of individual motor axons changes during synapse elimination. Moreover, little is known about the advancement of synapse elimination at one neuromuscular junction relative to other junctions in the same motor unit. In particular, it remains unclear what factors determine which branches of an axon are lost and when. A better understanding of the details of branch removal should provide insights into the driving force behind synapse elimination and what factors generate the final pattern of axon branching within a muscle and perhaps elsewhere in the nervous system.

To directly study motor axon branch reorganization during the period of synapse elimination, we have utilized lines of transgenic mice that express a fluorescent protein (YFP or GFP) in only one or a few of the many motor axons that innervate each muscle (Feng et al., 2000). Confocal three-dimensional reconstructions of

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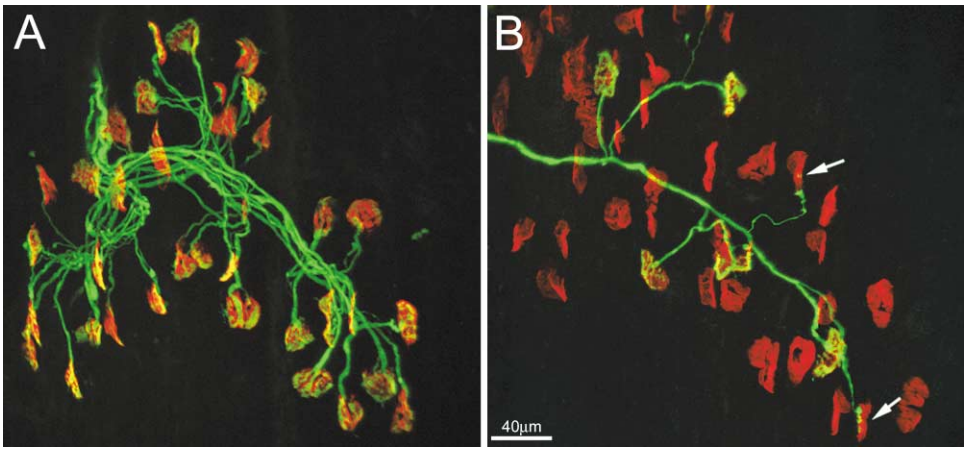

Figure 1. YFP Is Expressed in All or Only One Motor Axon Projecting to a Muscle in Different Transgenic Lines

(A) Confocal reconstruction of part of the band of neuromuscular junctions in a sternomastoid muscle from a P8 *thy1-YFP* line F mouse. Note that the YFP containing axons (green) project to all junctions and completely occupy the high-density AChR clusters (red, labeled with rhodamine α-bungarotoxin), indicating that all the motor axons express YFP.

(B) Confocal reconstruction of part of the band of neuromuscular junctions in a P8 sternomastoid muscle from *thy1-YFP* line H mouse. In this muscle, only a single labeled axon was seen in the nerve, and its arbor was the only YFP labeling in the muscle (green). Note that many neuromuscular junctions (whose acetylcholine receptors are labeled with rhodamine) do not receive contact from the labeled axon and that several of the neuromuscular junctions contacted by the axon were only partially occupied (arrows).

labeled axons in these mice permitted visualization of the complete arbor of individual motor axons. Using this method, we analyzed the branching pattern of the entire motor unit in neonatal muscles and identified all of the postsynaptic partners within the muscle, during the period of synapse elimination. We report that axon branches are eliminated asynchronously within a single motor unit and that branch loss appears to be distributed randomly within the motor unit arbor. Thus, despite their identical activity patterns, the local competitive environment at each neuromuscular junction influences which branches will be maintained and which branches will be withdrawn.

Results

Transgenic Expression of Fluorescent Proteins in Subsets of Motor Neurons

Recently, we generated transgenic mice that express variants of green fluorescent protein (GFP) in the nervous system, under the control of regulatory elements from the *thy*1 gene (Feng et al., 2000). In most of the lines examined (21/25), all or nearly all motor axons and motor nerve terminals were labeled (Figure 1A). In four lines (YFP-H, GFP-S, GFP-M, and CFP-S), however, only small numbers of motor neurons were labeled within each muscle (Figure 1B). Each transgenic line had its own particular expression pattern. Moreover, in any one line, the exact number of motor axons expressing fluorescent protein within a particular muscle was somewhat variable. Possible reasons for these variations are discussed in Feng et al. (2000). From these transgenic lines, we chose two (YFP-H and GFP-S) in which small numbers of axons in the sternomastoid and spinotrapezius muscles expressed the reporter during the first 2 postnatal weeks when synapse elimination is occurring. Staining with neurofilament and synaptic vesicle antibodies in these transgenic mice showed that the subset of labeled axons was completely filled so that all their terminal branches and neuromuscular junctions were visible. Of the over 800 sternomastoid and spinotrapezius muscles from YFP-H and GFP-S mouse lines examined at postnatal day 8 (P8), there were none in which more than two motor axons were labeled, and, most often, no axons were labeled. For example, in the sternomastoid muscle ($n = \sim 500$), two motor axons expressed fluorescent protein in $<1\%$ of the muscles examined, one axon in 10% of muscles, and no axons in 90% of muscles; in the spinotrapezius ($n = \sim 300$), two axons in $<1\%$ of muscles, one axon in 35% of muscles, and no axons in 65% of muscles. In these same lines, nearly all muscles were labeled in older animals, and more axons were labeled per muscle (~ 7 in the sternomastoid and ~ 12 in the spinotrapezius by P40). These mice provided the first opportunity to visualize the branching patterns of single axons in vertebrate muscle.

Partial Occupation of Neuromuscular Junctions by Labeled Axons

We surveyed the size and disposition of motor units at several ages during the first 2 postnatal weeks. At postnatal day 8, in both the sternomastoid and spinotrapezius muscles, single YFP-labeled motor axons could be seen to branch and contact many neuromuscular junctions. Frequently, however, the terminal branches innervated only a portion of the individual neuromuscular junctions they contacted (Figures 1B and 2A). In Figure 2A, for example, branches of a single motor axon innervate four nearly adjacent muscle fibers, but only two of the neuromuscular junctions (far left and far right) are completely or nearly completely occupied by the labeled axon. In contrast, the acetylcholine receptors (labeled red with rhodamine-tagged α-bungarotoxin) of the upper and lower junctions (asterisks) are partially

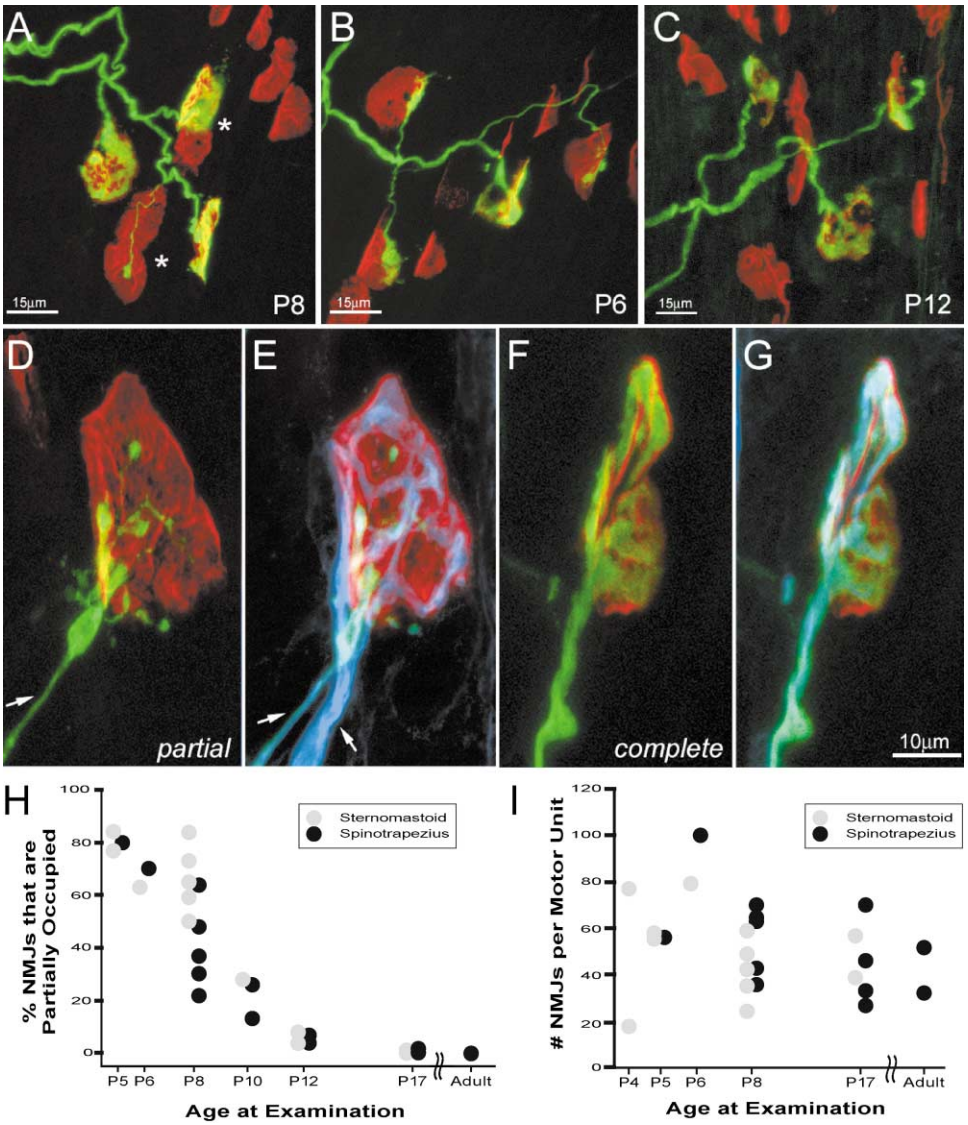

Figure 2. Fluorescently Labeled Motor Axons Partially Occupy Some Neuromuscular Junctions that Are Also Innervated by Other Axons in Early Postnatal Life

(A) Confocal reconstruction of the contacts of one YFP-labeled axon (green) on four neuromuscular junctions (AChRs labeled red) in the sternomastoid muscle at P8. Two of the junctions are partially occupied by the labeled axon (asterisks), and the other two appear completely occupied.

(B) Reconstruction of a portion of a P6 YFP-labeled motor unit in the spinotrapezius muscle shows more junctions that are partially occupied than in older animals.

(C) Reconstruction of part of a P12 YFP-labeled motor unit in a spinotrapezius muscle. At this age, labeled axons such as the one shown occupy all or almost all of the junctions they contact.

(D) A neuromuscular junction from a sternomastoid muscle in a P8 *thy1-GFP* line S mouse. Note that the AChRs (red) are only partially occupied by the labeled axon (green, arrow).

(E) The same junction as shown in (D), immunolabeled with neurofilament and synaptic vesicle antibodies (blue). Note that a second thicker axon (blue, arrow) also innervates this junction and occupies AChRs that are unoccupied by the GFP-labeled axon.

(F) A single neuromuscular junction from a sternomastoid muscle in a P8 *thy1-GFP* line S mouse that is completely occupied by a GFP-labeled axon.

(G) The same junction shown in (F), labeled with antibodies to neurofilament and synaptic vesicles (blue). Note that no additional axons innervate the junction, indicating that it is singly innervated and therefore has completed synapse elimination.

(H) Graph showing the percentage of neuromuscular junctions in a motor unit that are partially occupied (i.e., multiply innervated) as a function of age. Each circle represents one motor unit: gray circles are sternomastoid motor units, and black circles are spinotrapezius motor units. Note that the number of junctions that are partially occupied decreases as the animal matures.

(I) Graph showing the number of neuromuscular junctions (based on confocal reconstructions) in a motor unit as a function of age. Each circle represents one reconstructed motor unit: gray circles are sternomastoid motor units, and black circles are spinotrapezius motor units.

occupied by branches of the labeled axon. Neuromuscular junctions with partial occupation of the receptor areas were seen in every P8 motor axon studied in detail ($n > 60$) and in several different muscles, including the sternomastoid, spinotrapezius, anterior serratus, gluteus, and diaphragm (see below).

We also examined branches of motor units at younger and older ages. In younger animals, labeled motor axons were more apt to partially occupy neuromuscular junctions, and, in older animals, partial occupation was rare. For example, in the spinotrapezius muscle at P6, 70% of the neuromuscular junctions contacted by a labeled motor axon were partially occupied ($n = 103$ junctions in two animals). At P8, however, labeled axons in the spinotrapezius partially occupied only 40% of the junctions they contacted ($n = 277$ junctions in five animals). At P10, labeled axons partially occupied 20% of the junctions they contacted ($n = 57$ junctions in two animals), and, by P12, only 4% of the spinotrapezius neuromuscular junctions were partially occupied by a labeled axon ($n = 74$ junctions in two animals). In animals that had completed the major phase of synapse elimination (P17), less than 1% of the neuromuscular junctions were partially occupied ($n = 176$ junctions in four motor units). In adults, partial occupation was not seen ($n = 84$ junctions in two motor units).

By immunolabeling all axons in a muscle with anti-neurofilament antibodies, we found that junctions partially occupied by a YFP- or a GFP-expressing axon were always additionally contacted by a second and in some cases a third innervating axon (Figures 2D and 2E). Conversely, junctions that appeared completely occupied by the labeled axon were singly innervated (Figures 2F and 2G). Thus, we could infer which junctions had not completed synapse elimination by viewing the receptor territories occupied by a labeled axon at each neuromuscular junction. The decrease in partially occupied junctions observed between P5 and later ages thus reflects the loss of multiple innervation. The plot of the incidence of partially occupied neuromuscular junctions as a function of age (Figure 2H) is sigmoidal in shape. This curve is the same as those obtained assaying multiple innervation by other measures, including intracellular recording and axon counts (see, for example, Balice-Gordon and Lichtman, 1993; Bennett and Pettigrew, 1974; Brown et al., 1976).

Axonal Atrophy and Branch Withdrawal

Interestingly, axon branches contacting the smallest proportion of receptors at neuromuscular junctions were typically the thinnest terminal branches. For example, in Figure 2A, the lower junction is partially occupied by an axon that overlies a very small receptor area. Correspondingly, this terminal branch is substantially smaller than other branches of the same motor axon that occupy relatively larger proportions of receptor areas. Systematic analysis of axon diameter as a function of receptor occupation (see below) showed generally that diameter decreases as occupation decreases. Because synapse elimination is thought to be progressive at each neuromuscular junction (Balice-Gordon et al., 1993; Colman et al., 1997; Kopp et al., 2000), the proportion of a junction occupied by an axon may be an indication of the progress of synapse elimination and of the likelihood that the axon branch will be trimmed. Once an axon completely retracts from a neuromuscular junction, it is typically of very thin caliber and ends in a retraction bulb (Bernstein and Lichtman, 1999).

In order to test the idea that thin branches are in the process of retracting, we imaged superficial neuromuscular junctions innervated by YFP-labeled motor axons (100% axons expressing YFP) in living mice during the period when synapse elimination removes axonal branches. The example shown in Figures 3A–3C shows three views of the same two neuromuscular junctions at P7, P8, and P9, respectively. At P7, branches of two different YFP-labeled axons innervate the junction on the left. The junction on the right is already singly innervated. Its innervation is provided by an axon that bifurcates (asterisk, Figure 3A) to innervate both of these junctions. One day later (P8), one of the branches innervating the left junction has become thin (arrow, Figure 3B). The sibling branch innervating the neighboring junction is unchanged. Over the next 24 hr, the atrophic branch has retracted from the left junction and now terminates in a retraction bulb (arrow, Figure 3C). These images suggest that atrophic axons that coinnervate neuromuscular junctions may be trimmed. Conversely, junctions that are singly innervated (see, for example, the right junction in Figures 3A–3C) are likely to be stably maintained. Less clear is the ultimate fate of branches that occupy some fraction of the junctional area and are not thin (e.g., upper junction in Figure 2A).

Reconstruction of Whole Motor Units Labeled with YFP

In a number of muscles, we studied all the terminal branches of individual labeled motor axons by montaging confocal image stacks. Eighteen motor units from animals between P4 and P8 were completely reconstructed; an additional 26 motor units were partially reconstructed between P6 and P17. Examples of completely reconstructed motor units at P8 are shown in Figure 4. In all cases, motor axons projected to a circumscribed subregion of the endplate band, leaving large numbers of receptors completely out of the territory of the labeled axon. In the sternomastoid muscle, motor axons entered the muscle centrally and branched to either the medial or lateral half of the muscle with no motor axon contacting neuromuscular junctions on both halves of the muscle (Figures 4A and 4B). Each sternomastoid motor unit was distributed over approximately 25% of the length of the endplate band, where it innervated a small proportion of the neuromuscular junctions (e.g., 35/288 or 12% of the nearby junctions for the motor unit shown in Figure 4A), suggesting that a subset of motor units, of which there are probably $\sim$35 to 60 in total (Nguyen et al., 1998), must project to this region.

In the subset lines in which only one or two axons were labeled in the sternomastoid, the projection pattern varied from muscle to muscle. For example, the labeled axon sometimes branched into a medial fork of the nerve, and, sometimes, it branched into a lateral fork. All the labeled motor axons in the sternomastoid did, however, have certain features in common. They tended to have a main, large-caliber trunk line, with multiple

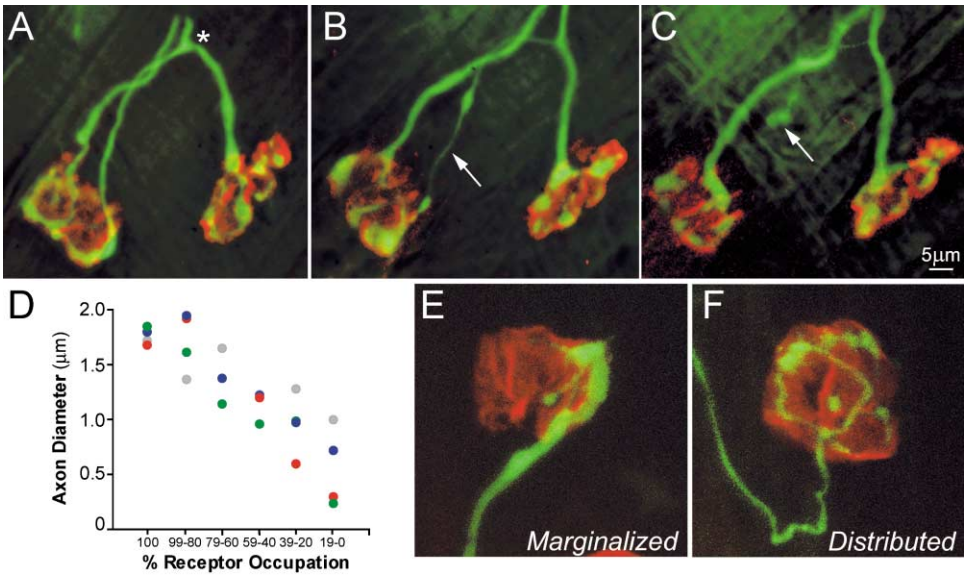

Figure 3. Withdrawing Axon Branches Atrophy

(A–C) Two neighboring neuromuscular junctions innervated by YFP-labeled motor axons were imaged in living mice 3 days in a row.

(A) At P7, the left junction is multiply innervated by two different YFP-labeled axons, while the right junction has already completed elimination and is singly innervated. Note that the right junction is innervated by an axon that bifurcates (asterisk) and also innervates the left junction.

(B) Just 1 day later at P8, one of the two inputs to the left junction has atrophied (arrow). The sibling branch innervating the neighboring junction is unchanged.

(C) At P9, the atrophic branch has withdrawn from the left junction and now ends in a bulb (arrow).

(D) Graph showing the caliber of terminal axon branches innervating junctions as a function of the percentage of junctional AChRs occupied in motor units at P8. Green and red circles are the mean diameter of axon branches from two P8 spinotrapezius motor units. Blue and gray circles are data from two P8 sternomastoid motor units. The junctional areas were binned into 20% increments, and all the axons within each group were averaged to obtain the plotted diameters. Note that axons that occupy the smallest proportion of receptors also have the smallest diameter axons.

(E) Postnatal day 8 neuromuscular junction showing an axon that partially occupies AChRs and is segregated to the right margin of the junction.

(F) Postnatal day 8 neuromuscular junction from the same motor unit as (E), showing an axon that partially occupies the junctional AChRs but, in contrast to the junction shown in (E), is distributed relatively uniformly across the junction. Because axons are thought to segregate prior to completion of synapse elimination, these two axon branches from the same neuron (E and F) are likely at different stages in the synapse elimination process.

smaller branches coming off the trunk. In the one case in which we found two motor axons expressing YFP in the same neonatal sternomastoid muscle (see drawing, Figure 4B), the two axons both projected to the same fork but terminated in nonoverlapping territories. P8 sternomastoid motor units contacted an average of 42 $\pm$ 12 muscle fibers (n = 6). Unlike the transverse band of neuromuscular junctions in the sternomastoid, in the spinotrapezius muscle, the junctions are oriented more longitudinally (Figure 4C). Similar to the sternomastoid, however, motor units in the spinotrapezius muscle had a single thick central trunk, with various small-caliber branches. As with sternomastoid motor axons, labeled spinotrapezius axons were spatially restricted, deploying all their innervation in only a portion of the muscle. P8 spinotrapezius motor units contacted an average of 55 $\pm$ 15 muscle fibers (n = 5).

Motor units in P6 animals differed from those at P8 mainly in size; they were significantly larger ($\sim$2-fold, p = 0.004; Figures 2I and 4D and Table 1). The larger size of motor units at P6 compared to P8 suggests that axon branches are trimmed rapidly between these times, which is consistent with the steep slope in the reduction of polyneuronal innervation over the same time period (see Figure 2H and Figure 1 in Balice-Gordon and Lichtman, 1993). We also examined the size of motor units in P4 and P5 animals. The incidence of singly labeled axons in YFP-H and GFP-S lines drops precipitously at ages below P8. Nonetheless, from approximately 20 litters (150 neonatal animals), we found three P5- and two P4-labeled motor axons. Interestingly, the five motor units reconstructed from these animals were not, on average, larger than their older counterparts (Figure 2I). This may reflect a certain amount of branch addition during the first postnatal week if new muscle fibers are added to this muscle, as has been observed in other rodent muscles (for example, rat lumbrical muscle; Betz et al., 1979). Motor units in early development are also thought to vary more widely in twitch tension compared to later (Brown et al., 1976); the reasons for this variance are not presently understood.

In contrast to the decrease in size of motor units between P6 and P8, we did not detect a significant decrease in motor unit size between P8 and P17 (Figure 2I). This likely reflects the fact that, in some motor units at P8, more than 70% of their neuromuscular junctions are singly innervated, and the motor units have therefore completed the bulk of synapse elimination.

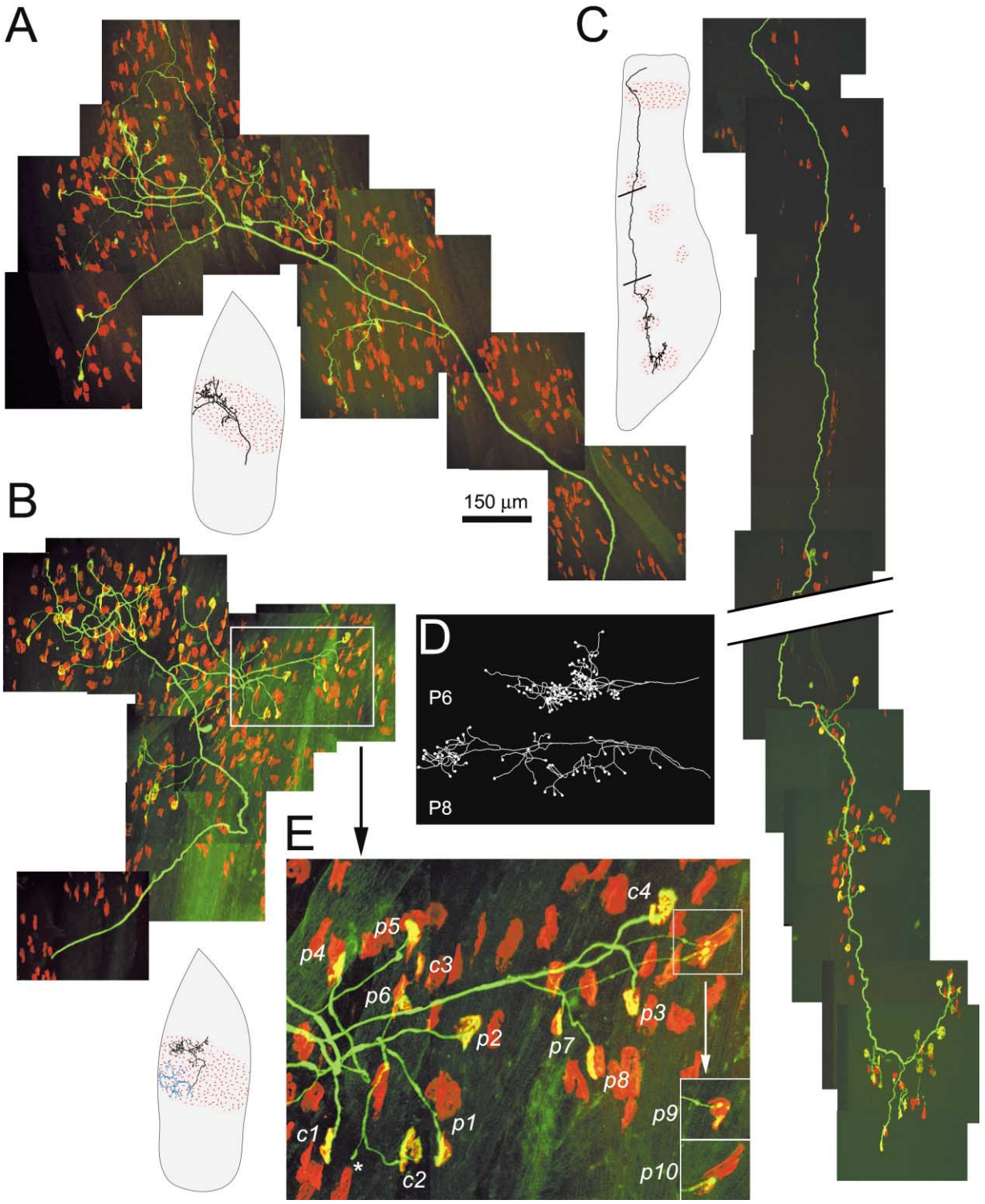

Figure 4. Completely Reconstructed Motor Units in the Sternomastoid and Spinotrapezius Muscles

(A) A YFP-labeled motor unit in the sternomastoid muscle at P8. This motor axon (green) contacted 35 neuromuscular junctions, approximately 12% of the receptor sites (red) in that portion of the muscle. The territory circumscribed by this motor unit is shown in the small drawing below.

(B) Another P8 motor unit in a sternomastoid muscle, which contacts 49 neuromuscular junctions. In this muscle, there were two labeled motor axons. The small drawing below shows the location of the two motor units; the reconstruction shown is the upper axon in the drawing (black ink). The two motor units are each confined to the same half of the muscle but do not overlap. The white box highlights a region of the motor unit shown at higher magnification below (black arrow, Figure 3E).

(C) Montage of a P8 spinotrapezius motor unit. Unlike the laterally spreading motor units in the sternomastoid muscle, spinotrapezius motor axons run vertically in the long axis of the muscle. This motor unit contacted 36 neuromuscular junctions. Some of the contact sites (e.g., the top one shown) were quite far (~7 mm) from the majority of innervated junctions.

Table 1. Motor Units in Neonatal Mouse Muscles

	Number of Muscle Fibers Contacted	Number of Singly Innervated (%)	Number of Multiply Innervated (by % Receptor Occupation)			
			≥75%	75%–25%	≤25%	Unknown
Sternomastoid						
P5 #1	57	9 (16)	17	21	10	0
P5 #2	57	13 (23)	18	18	8	0
P6 #1	79	29 (37)	15	32	3	0
P8 #1	43	7 (16)	12	16	8	0
P8 #2	24	12 (50)	4	8	0	0
P8 #3	49	20 (41)	6	19	4	0
P8 #4	35[a]	9 (27)	5	13	6	0
P8 #5	59	21 (36)	9	19	8	2
Spinotrapezius						
P5 #1	56	11 (20)	13	22	10	0
P6 #1	100	30 (30)	17	36	17	0
P8 #1	43	30 (70)	3	7	3	0
P8 #2	36	28 (78)	3	2	3	0
P8 #3	65	41 (63)	9	12	3	0
P8 #4	63	33 (52)	11	11	8	0
P8 #5	70	25 (36)	12	20	13	0

[a] Includes two β endings (α motor neuron contacts on intrafusal fibers).

Temporal Aspects of Synapse Elimination

In the first 2 postnatal weeks, we found evidence showing that different motor units undergo most of their branch withdrawal at different times. For example, at P8 in the sternomastoid muscle, motor units ($n = 5$) ranged nearly 3-fold in terms of the percentage of their axon branches that singly innervated neuromuscular junctions (i.e., terminal branches that completely occupied junctions). At one extreme, we found a sternomastoid motor unit with only 16% singly innervated junctions, whereas another had 50% singly innervated junctions. In the spinotrapezius at the same age, motor units ($n = 5$) also showed a wide range (36%–78% singly innervated). These results suggest that different motor units in a muscle complete the branch withdrawal process at different times. In addition, on average, we found that the spinotrapezius muscle was further along in completing synapse elimination than the sternomastoid; at P8, 60% of the neuromuscular junctions were singly innervated in spinotrapezius motor units ($n = 5$) compared to 34% singly innervated neuromuscular junctions in sternomastoid motor units ($n = 5$, $p < 0.05$). This intermuscle variation is consistent with previous work suggesting the rate of synapse elimination differs between muscles (Bixby and VanEssen, 1979).

Several results argue that synapse elimination is proceeding asynchronously within a motor unit. First, because individual axons had branches that simultaneously singly innervated some junctions and coinnervated others, branch trimming is not occurring at the same time in all branches. Second, the proportion of each neuromuscular junction occupied by a labeled motor axon varied considerably from one junction to another, suggesting that different junctions may be at different stages in the process. For example, in the high-magnification image from the P8 sternomastoid motor unit shown in Figure 4E, there are 14 neuromuscular junctions innervated by the YFP axon; four of them appear to be completely occupied by the labeled axon (c1–c4), ten are partially occupied (p1–p10), and one additional junction is adjacent to a thin labeled axon branch ending in a retraction bulb (asterisk), suggesting that this junction recently lost innervation from the labeled axon. Three junctions (p1–p3) are largely (>75%) but not completely occupied by the labeled axon. Another five junctions (p4–p8) are 25%–75% occupied by the labeled axon. The two remaining partially occupied neuromuscular junctions (p9–p10) are weakly contacted (<25%) by the labeled axon. Interestingly, the weakest contacts are associated with small-caliber axon branches. This trend was evident when looking at all the junctions within a motor unit and ranking them in terms of the proportion of junctional area occupied by the axon (Figure 3D and, for example, see left panels of Figures 5 and 6). When the axon occupied all or a large proportion of the AChRs, the axon branch tended to be thick, and, when the axon innervated a minority of the AChRs, it tended to be thin.

Finally, asynchronous synapse elimination is suggested by the observation that, within a motor unit, some of the endings appeared to be highly skewed to occupy only one margin of the neuromuscular junction, whereas, at the same time, other branches terminated in a more distributed way (compare Figures 3E and 3F). The presence of inputs occupying one margin is more common in older neonatal animals, suggesting that it is a relatively late step in synaptic competition (Gan and Lichtman,

(D) Drawings of two sternomastoid motor units: one at P6 (top) and one at P8 (bottom). Both drawings are at the same magnification. Small white circles show each neuromuscular junction. The P6 motor unit innervates 79 junctions, and the P8 motor unit innervates 43 junctions. (E) This high-magnification region of the motor unit in Figure 3B shows axon branches contacting 14 junctions and one retraction bulb (asterisk). Four of the contacted junctions appeared completely occupied by the labeled axon (c1–c4), and ten of the junctions were partially occupied (p1–p10). Two of the partially occupied junctions (p9 and p10) overlapped in the z axis and are shown separately (white arrow). Both of these axon branches were thin and occupied only a small proportion of junctional AChRs.

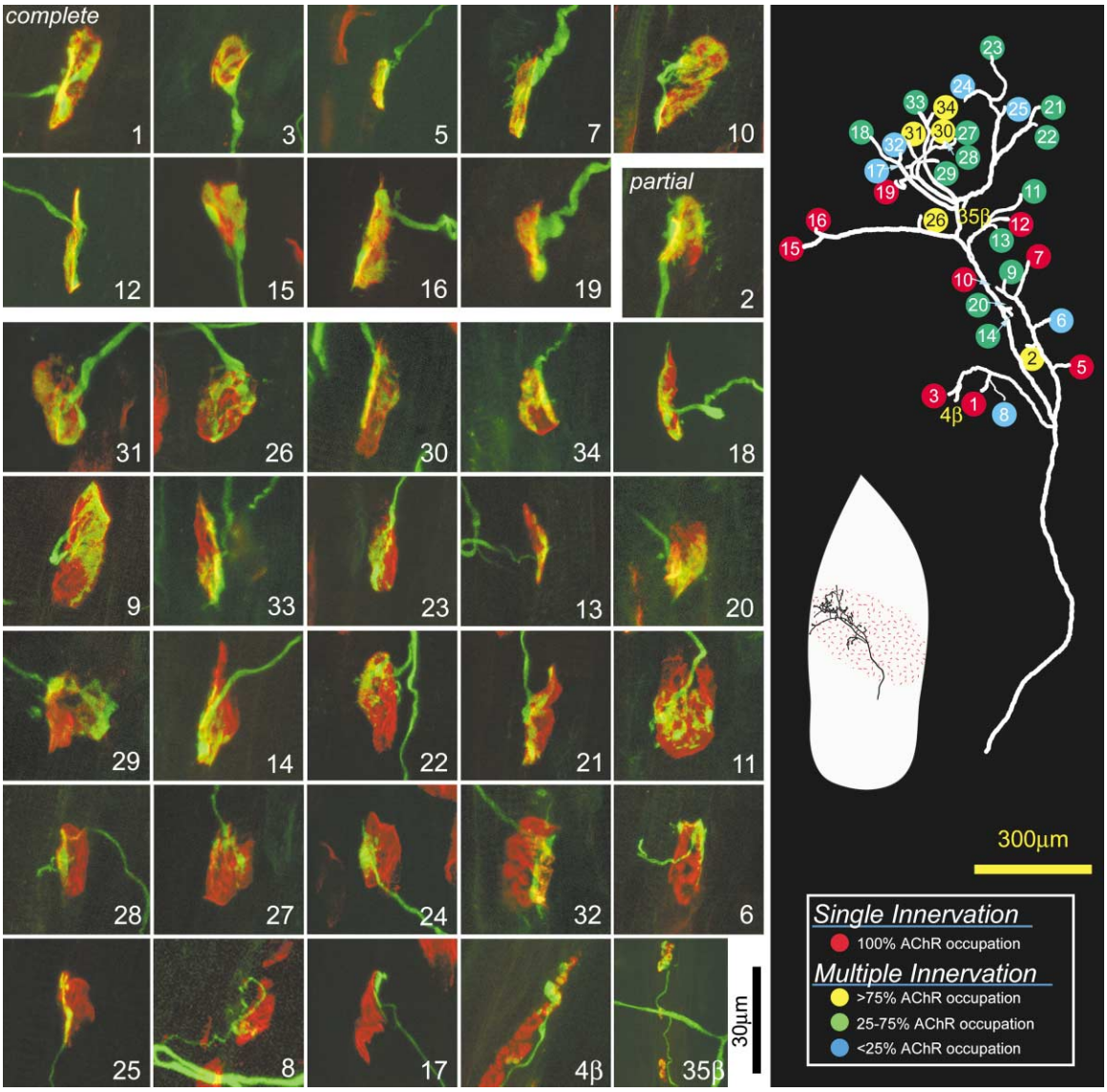

Figure 5. Reconstructions of the Entire Cohort of Neuromuscular Junctions in a Single Neonatal Sternomastoid Muscle Motor Unit

(Left) Neuromuscular junctions in this P8 sternomastoid motor unit (shown in Figure 4A) were arranged in order, with junctions that were completely occupied by the labeled axon shown on top and junctions with progressively less area of occupation shown offset below. The last two images are β endings on muscle spindles. The number in the bottom right of each junction's image refers to its location in the drawing of the arbor shown to the right. (Right) Shown is a drawing depicting the location of junctions with various amounts of AChR occupation. The smaller drawing shows the location of the motor unit in the muscle. The colored circles indicate the degree of junctional occupation (see text and key at bottom of figure). This motor axon contacts 35 junctions, and 27% of the junctions appear to have completed synapse elimination.

1998). For example, in the P8 sternomastoid motor unit reconstructed in Figure 5, several of the partially occupied neuromuscular junctions were highly marginalized (e.g., junctions 23, 14, 22, 24, 6, and 17), whereas others are more distributed (e.g., junctions 26 and 11). The axon branches that are not completely marginalized also only occupy some of the receptor sites in the territory where their branches reside. These distributed endings probably interdigitate extensively with other axonal branches that occupy the same parts of the junction (see, for example, Figures 2D and 2E). Similar results were evident in each of the motor units analyzed in this way (see, for example, Figure 6). The tendency for both types of endings to be seen at the same time suggests that the progress of synapse elimination is staggered among branches of a single motor axon.

Spatial Aspects of Synapse Elimination

Motor unit trimming during synapse elimination might be regulated by regional factors. For example, certain parts of an axonal arbor might be involved in the synapse elimination process at an earlier time than other parts, or certain parts of an axonal arbor might have a greater tendency to be trimmed than other parts. In either case, there should be some detectable spatial apportionment of singly and multiply innervated junctions in a motor

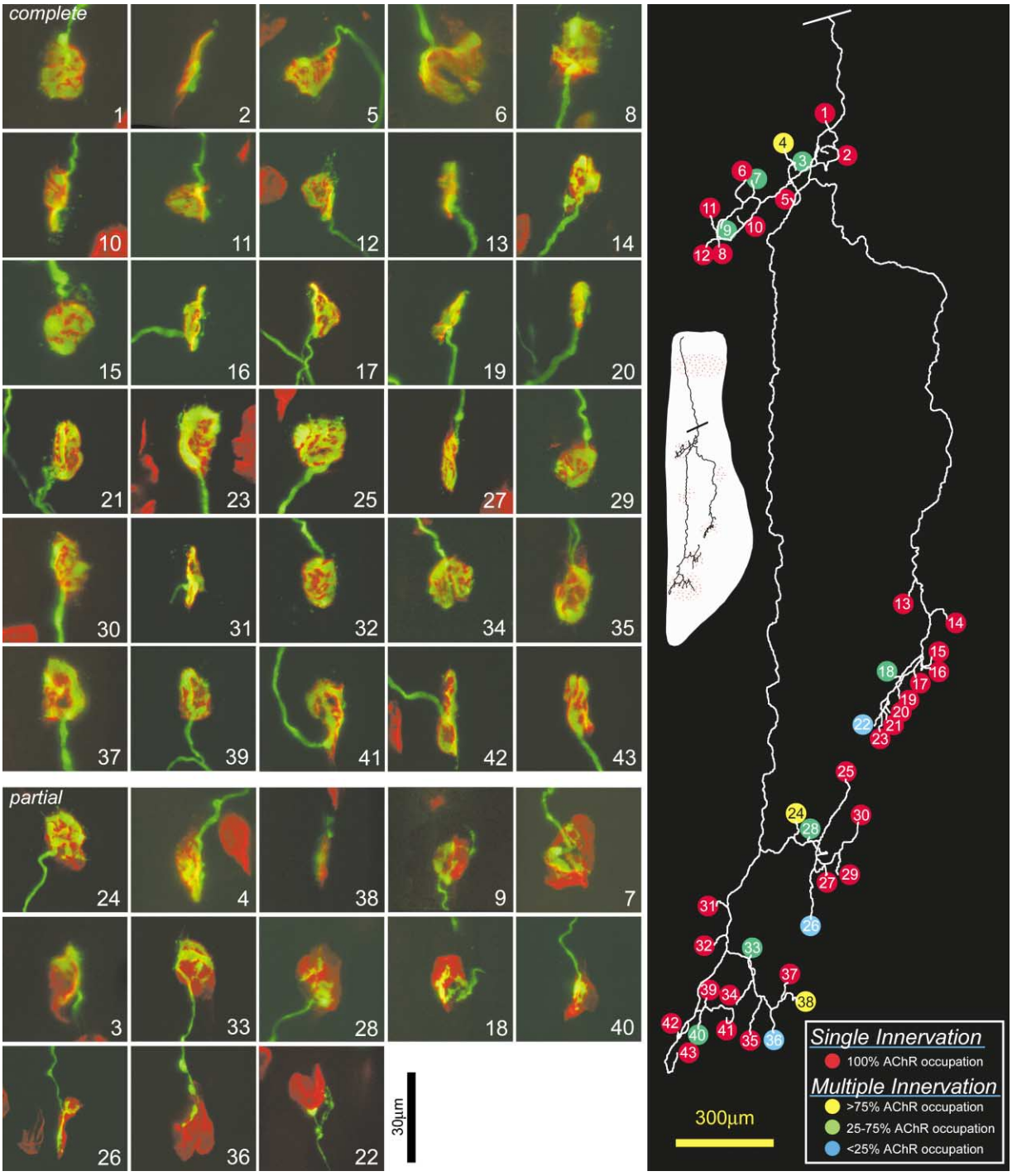

Figure 6. Reconstructions of the Entire Cohort of Neuromuscular Junctions in a Single Neonatal Spinotrapezius Muscle Motor Unit

(Left) Neuromuscular junctions in this motor unit were arranged in order, with junctions that were completely occupied by the labeled axon shown on top and junctions with progressively less area of occupation shown offset below. The number in the bottom right of each junction's image refers to its location in the drawing of the arbor shown to the right. (Right) Shown is a drawing depicting the location of junctions at various stages in the synapse elimination process relative to their position in the motor unit. The smaller drawing shows the location of the motor unit in the muscle. The colored circles indicate the degree of junctional occupation (see text and key at bottom of figure). The P8 spinotrapezius motor axon shown contacts 43 neuromuscular junctions, and 70% of them appear to have completed synapse elimination.

unit. To test this idea, we analyzed 15 fully reconstructed motor units: ten motor units at P8, two motor units at P6, and three motor units at P5 (data in Table 1).

These data were analyzed three ways. First, we asked if the location of a terminal branch within the muscle affected the outcome of synapse elimination. Because

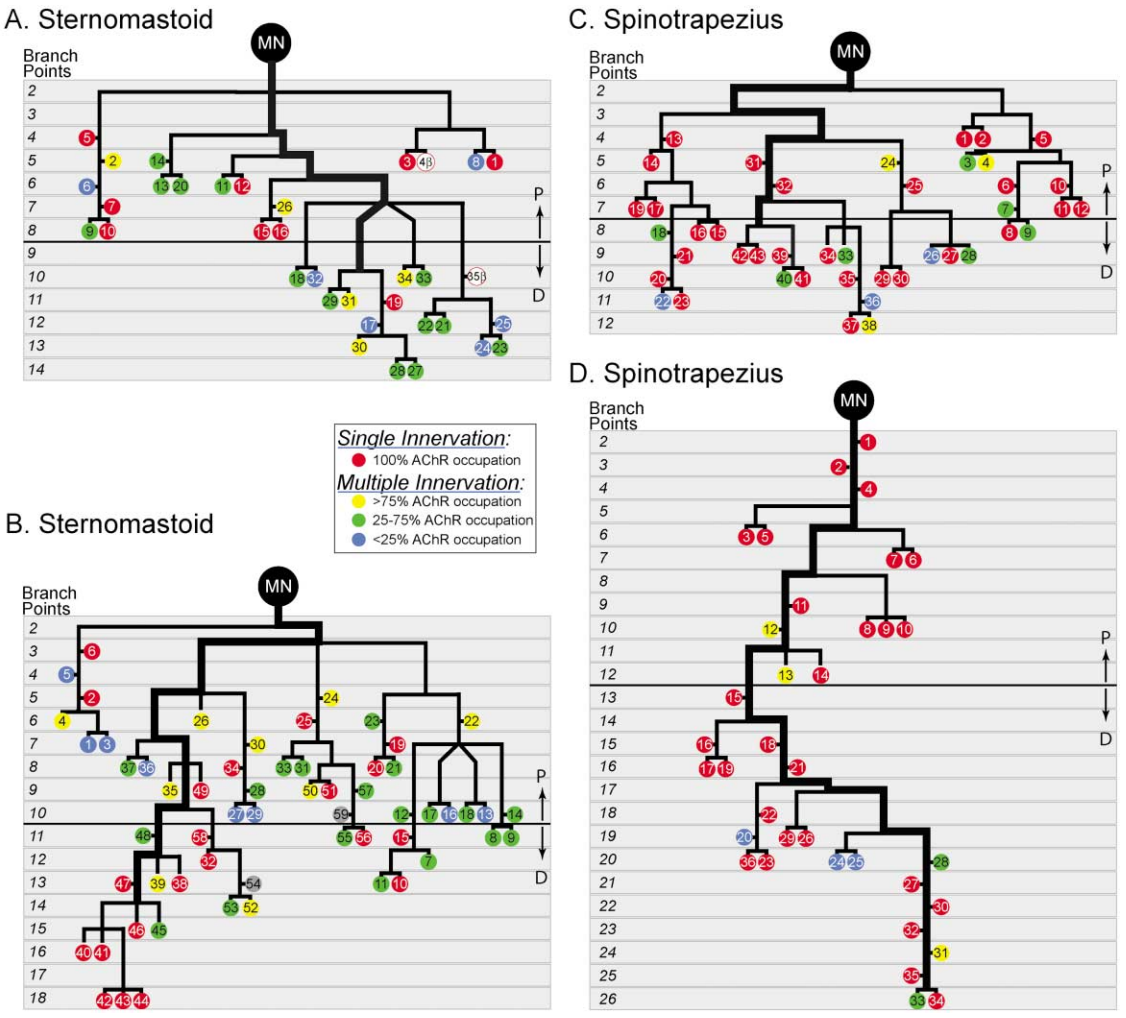

Figure 7. Complete Branching Diagrams of Neonatal Motor Units

(A) A sternomastoid motor unit (Figures 4A and 5 and P8 #4 in Table 1).

(B) A sternomastoid motor unit (P8 #5 in Table 1).

(C) A spinotrapezius motor unit (Figure 6 and P8 #1 in Table 1).

(D) A spinotrapezius motor unit (Figure 4C and P8 #2 in Table 1). Note that in this motor unit, the most distal branches (i.e., branch point 26) are not the caudalmost branches in the muscle, because, after coursing caudally, the nerve curves back rostrally (see, for example, Figure 4C). The stage of synapse elimination at each junction is color coded in the same manner as before (see key in figure). Gray circles indicate junctions at an undetermined state of occupancy. The thicker black lines denote the trunk line of the axon. The number of branch points between each neuromuscular junction and the cell body is shown to the left of each diagram. P and D refer to proximal and distal branches. The reconstructed motor units in the sternomastoid muscle had terminal branches ending in neuromuscular junctions between 3 and 18 branch points from the cell body (mean = 9.5 branch points, n = 2). In the spinotrapezius muscle, neuromuscular junctions ranged from 2 to 26 branch points from the cell body (mean = 11.4 branch points, n = 2).

the reconstructions came from confocal stacks, we could analyze position in all three dimensions. Singly innervated neuromuscular junctions (see red circles, Figures 5 and 6) appeared randomly distributed throughout much of the motor unit's territory. Thus, there was no tendency for the medial, lateral, rostral, caudal, superficial, or deep regions of the motor unit to be dominated by junctions that have completed the process of synapse elimination. Similarly, junctions that were innervated by branches that were thin and/or occupied less than 25% of the receptors (blue circles, Figures 5 and 6) were also distributed throughout the motor unit's territory without any obvious pattern. Thus, despite the fact that the motor unit is found in a circumscribed part of the muscle, within that region there is no evidence that motor axons prefer one territory more than another. Nor is there evidence that synapse elimination is completed in one part of the territory earlier than in another part.

Second, we asked if the relative position of a terminal branch with respect to other branches within the same motor unit had an impact on the ultimate fate of that branch. For example, terminal branches that originate early in the branching scheme (proximal branches) might have access to more resources (and thus be more likely to be maintained) than branches that are far down in the branching pattern (distal branches). In order to make this analysis, we constructed complete branching dia-

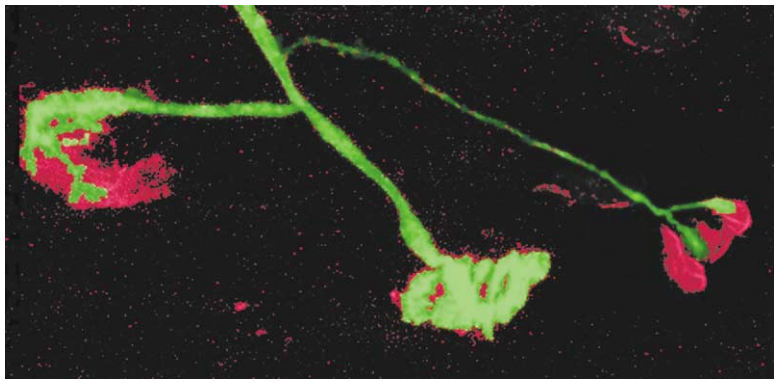

Figure 8. DiO-Labeled Axon at P7 Shows Terminal Branches from a Single Motor Unit that Appear to Be at Different Stages of Synapse Elimination

The DiO-labeled axon is green, and the AChRs are red (labeled with rhodamine α-bungarotoxin). Shown are three junctions contacted by the same axon. The left branch occupies roughly half the AChRs, the center branch appears to occupy the entire junction, and the right terminal branch contacts a small proportion of the receptor region by a thin, atrophic branch.

grams for some of the motor units described above (examples are shown in Figure 7). In each diagram, a large-caliber main trunk (see above) is designated by a thicker black line, and colored circles (as in Figures 5 and 6) indicate each synapse.

These branching diagrams showed that there was no simple branching plan; most branch points were bifurcations (91%), but there were a few trifurcations (8%) and two sites where motor axons divided into four branches (1%). Moreover, 48% of the bifurcations were asymmetrical: giving rise to one terminal branch and one branch that divided one or more times subsequently. The symmetrical branches (52%) also varied, with 58% of them having two terminal branches and 42% in which both branches divided again.

We found no evidence suggesting that proximal branches were, on average, any different from distal branches in terms of progress toward single innervation. Of the 85 terminal branches that were in the proximal half of the four motor units shown in Figure 7, 42 (50%) had already completed synapse elimination (red circles). Similarly, 46 of 88 distal branches (52%) had completed synapse elimination. Likewise, there did not seem to be any intrinsic tendency for thin branches or branches that occupied small proportions of the receptors (blue circles) to be skewed to the proximal or distal halves. Of the 85 proximal branches, 10 (12%) occupied a small amount of territory. Similarly, 10 of 88 distal branches (11%) occupied a small amount of the junctional area. From these results, we conclude that the time of completion of synapse elimination was not influenced by the proximal or distal location of an axon branch.

Finally, pairs of neuromuscular junctions that were innervated by a bifurcation of the same labeled motor axon were analyzed to determine whether there was any correlation between the stage of synapse elimination of one terminal branch relative to its sibling branch. Each terminal branch of a pair was categorized in terms of its junctional receptor occupation (i.e., 100%, $>$75%, 25%–75%, $<$25%). In both the sternomastoid and spinotrapezius motor units, there was no significant trend for sibling branches to either occupy the same amount of territory or occupy different amounts of territory. The proportion of junctions in each category was what would be expected by chance, arguing that the process of synapse elimination as it occurs at one branch was independent of the process as it occurred at the sibling branch. An example of two sibling branches with opposite outcomes is shown in Figures 3A–3C. Taken together, the results show that, in general, there is little evidence of regional control of synapse elimination and therefore suggest that synapse elimination at each neuromuscular junction is dominated by local interactions between terminal branches of different axons that are in competition.

Single Motor Units Labeled with DiI

To test the possibility that YFP labeled a nonrepresentative set of motor axons or that YFP expression affected synapse elimination, we analyzed the branching patterns of axons labeled in a different way. Single motor axons were labeled by iontophoretic application of lipophilic dyes from sharp electrodes (Gan et al., 1999) during the first 2 weeks of postnatal life. The dye placement technique provided a clear view of several branches of the same axon (Figure 8). Branches labeled in this manner showed the same kind of variation we had seen in the transgenic mice. In Figure 8, for example, a DiO-labeled motor axon branches to innervate three junctions on three muscle fibers at P7. The occupation area varied among these three junctions. The junction in the middle is occupied completely, or almost so, by the labeled axon. The junction on the left is about half occupied by the labeled axon, and only a small amount of the junction on the right is occupied by the labeled axon. Correspondingly, the axon calibers are also different; the branch occupying the smallest proportion of a neuromuscular junction had the smallest caliber. Similar variations were seen in all labeled muscles ($n = 6$) and suggest that the results obtained with YFP-labeled axons were not anomalous.

Discussion

Reconstruction of Entire Motor Units

We have examined naturally occurring remodeling of axonal arbors in early postnatal life. Our analysis was made possible by the finding that, in some lines of transgenic mice expressing YFP or other GFP variants, only one or a few motor axons projecting to a muscle express fluorescent protein (Feng et al., 2000). This low level of expression gave us an unimpeded view of the complete branching pattern of an individual motor axon, as well as a method to identify the entire cohort of its postsynaptic targets (the motor unit).

We initially considered the possibility that the subsets

we observed represented particular identifiable motor neurons. Such identifiable neurons are commonplace in invertebrate nervous systems (Bullock, 2000). However, this possibility seems unlikely for several reasons. First, the expression seemed too stochastic to be revealing a particular neuron; in the sternomastoid and spinotrapezius muscles from P4–P8, there was a significant likelihood that no axons expressed fluorescent protein. Moreover, there was no correlation between expression in the ipsi- and contralateral muscles of the same mouse. Most importantly, it was not the case that labeled axons consistently projected to a particular region of the muscle (see Figure 4). The underlying reason for restricted expression in such a small number of axons remains unknown; several possibilities are discussed by Feng et al. (2000).

As mentioned above, these transgenic mice made it possible to reconstruct the entire branching pattern of a mammalian axon and identify all of its postsynaptic partners. These reconstructions revealed two unexpected features of motor axons. First, every motor unit we reconstructed projected to spatially discrete subregions of a muscle. In ongoing work, we are analyzing this spatial restriction to explore the idea that muscles are compartmentalized (Balice-Gordon and Thompson, 1988; Bennett and Lavidis, 1984; Gatesy and English, 1993) to a greater extent than previously thought. Second, motor axons generally have one main axonal branch that is of large caliber and is the source of numerous secondary branches. This "trunk line" may provide more distal axonal branches with greater access to resources originating from the cell body.

Axon Branch Trimming in Early Postnatal Life

Here, we focus on a third feature of the reconstructed neonatal motor units, which is that they provide direct confirmation of the idea that axons undergo branch retraction during development. Until this work, all evidence for branch retraction was inferential, based on physiological changes in the average twitch tension of motor units and stability in the counts of motor neurons in the spinal cord in early postnatal life—both of which were problematic techniques (Jansen and Fladby, 1990).

One conclusion of this work is that branch withdrawal occurs asynchronously within the arbor of a single motor axon. Previously, it was known that synapse elimination is asynchronous in the sense that individual neuromuscular junctions within the same muscle become singly innervated at different times during the first several postnatal weeks. For example, in the sternomastoid muscle at P0, all of the muscle fibers are multiply innervated, but, at P7, only half of the muscle fibers remain multiply innervated, and, by P14, only 1% of the fibers are still multiply innervated (Balice-Gordon and Lichtman, 1993). It was, however, not known whether this staggered transition from multiple to single innervation was also true for the neuromuscular junctions that comprise a motor unit. For example, it could have been that axon withdrawal by one motor axon was synchronized such that all of the postsynaptic partners from which it was withdrawing would be at the same stage in the process. Such synchrony would argue for some intrinsic neuronal control of branch withdrawal and provide support for the idea that axons undergo synapse elimination because of an intrinsic tendency to withdraw (Fladby and Jansen, 1987; Thompson and Jansen, 1977). Our results show, in contrast, that the withdrawal is not synchronized. In particular, at P8, terminal axon branches within the same motor unit were observed to be at different stages in the synapse elimination process. While some terminal branches provided the sole remaining innervation to junctions, other branches of the same axon partially occupied junctions that were still innervated by other axons. Furthermore, there was a wide range of appearances of neuromuscular junctions that were multiply innervated. For example, the diameter of the innervating axon, the percentage of the receptor territory occupied, and the physical location of the axon overlying the receptors (marginalized versus distributed) varied greatly at each junction within a single motor unit. Last, some terminal branches appeared to have recently retracted from muscle fibers, because they were no longer contacting postsynaptic receptors, were atrophic, and terminated in a bulb (see Bernstein and Lichtman, 1999). Therefore, within a single motor unit at a single point in time, it appeared that there were terminal branches that had completed the elimination process and survived, others that were still fighting for sole innervation rights, and some that had lost the competition and were retracting. Thus, despite the fact that one neuron propagates identical activity patterns to all of its branches, expresses one set of genes, and has a single spatiotemporal origin in the developmental milieu, its axon branches show great variation in their behavior.

Another conclusion is that the outcome of the branch withdrawal process does not seem to be affected by positional signals. It could have been that branch withdrawal was influenced by the location of the terminal branch within the muscle. For example, topographic cues in the muscle might drive motor unit remodeling (Bennett and Lavidis, 1984; Gatesy and English, 1993; Laskowski et al., 1998). Nor was the outcome of synapse elimination influenced by where the terminal branch was embedded in the motor unit's branching pattern. It could have been that neuromuscular junctions that are located fewer branch points from the cell body would have greater access to soma-derived materials. This proximity to the cell body might help these junctional branches maintain their connections to the muscle fiber. Finally, we found no evidence to suggest that synapse elimination at one branch necessarily influences the outcome at a sibling branch. Thus, many possible signals that could have produced nonrandom branch withdrawal do not appear to play a major role in arbor sculpting in the muscles studied.

Given our results, axonal branches of a single neuron are, to a large degree, undergoing synapse elimination independently. These results are consistent with the idea that each neuromuscular junction is a local arena of competition between different axons. In this view, both the outcome and rate of synapse elimination are dominated by the factors that determine the relative competitive vigor of the converging axons. What these factors are remain a matter of speculation, but relative differences between neurons in terms of activity patterns, protein expression, temporal characteristics (e.g., which axon's branch arrived first), and spatial character-

istics (e.g., which motor unit was less overextended elsewhere, and which terminal branch at the junction in question begins with a larger area of occupation) could determine the outcome. For example, it is possible that highly active axons undergo branch withdrawal earlier than axons that are relatively inactive (Barber and Lichtman, 1999).

One unexpected finding was that branches that occupied small areas of junctions almost invariably were thin compared to terminal branches of the same axons that occupied larger areas. It was previously known that branches that had already retracted (retraction bulbs) were associated with thinner branches (e.g., Gan and Lichtman, 1998). The time-lapse imaging of neuromuscular junctions shown here suggest that axons that will eventually be withdrawn undergo atrophy before they retract. This result thus argues against the idea that intrinsically thin axons withdraw because they are at a competitive disadvantage. The presence of thin branches in all motor units studied at P4–P8 argues that all motor axons are undergoing branch trimming during postnatal life.

In conclusion, transgenically labeled axons provide a new window on questions related to synaptic plasticity and remodeling. Until now, the focus has been almost entirely on the changes that occur at individual postsynaptic sites. Because it is now possible to analyze the entire postsynaptic population of synapses associated with one axon, many new and still uncharted questions about the behavior of axonal arbors can be addressed (see, for example, Keller-Peck et al., 2001). In this work, we find that decisions about which synapses will be maintained and which will be eliminated appear to have a very local component and suggest that the neuron cell body is not orchestrating the many ongoing competitions in which its branches are engaged.

Experimental Procedures

Mice

Transgenic mice (*thy1-YFP-H* and *thy1-GFP-S*) were generated as described previously (Feng et al., 2000), using *thy1* regulatory elements described by Vidal et al. (1990) and generously provided by P. Caroni (Caroni, 1997). *Thy1-YFP-H* mice, as well as two lines in which all motor axons are labeled (YFP-16 and CFP-23, Feng et al., 2000) are available from the Induced Mutant Resources at Jackson Laboratories (Bar Harbor, ME). Mice were mated to obtain timed pregnancies, and the day of birth was considered postnatal day 0 (P0).

Tissue Staining

Neonatal mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 2% paraformaldehyde in 0.1 M phosphated buffered saline (PBS, pH 7.4). Muscles to be used for DiI labeling were prepared according to previous methods (Gan et al., 1999). Transgenic XFP sternomastoid and spinotrapezius muscles were dissected, leaving several millimeters of the nerve attached to the muscle. The tissue was postfixed in paraformaldehyde for approximately 2 hr, the connective tissue was removed, and the muscles were incubated for 30 min at room temperature in 5 μg/ml tetramethylrhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) diluted in 1% bovine serum albumin (BSA) in sterile lactated Ringer's solution. In order to ascertain that single axons were labeled, the entire endplate band and several millimeters of attached nerve were examined. Muscles expressing fluorescent proteins in single axons were then mounted on slides in Vectashield (Vector Laboratories, Burlingame, CA) for imaging.

Muscles to be immunostained were blocked in 4% BSA and 0.4% Triton-X in PBS overnight at 4°C. The following day, tissue was incubated in mouse anti-phosphoneurofilament antibody SMI312 (Sternberger Monoclonals, Lutherville, MD) diluted 1:500 and the mouse synaptic vesicle antibody SV2 (Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA) diluted 1:10 in blocking solution. After washing 5 hr in PBS, muscles were incubated overnight in a 1:200 dilution of goat anti-mouse antibody conjugated to the fluorescent label Cy5 (Jackson ImmunoResearch). Muscles were then whole mounted on slides in Vectashield (Burlingame, CA) and imaged.

Imaging and Analysis

Single motor units and endplates were imaged on an Olympus (BX50WI) microscope using a laser scanning confocal microscope (BioRad 1024). Images were obtained with a 40$\times$ (1.35 NA) oil objective for reconstruction of whole motor units and a 100$\times$ (1.4 NA) oil objective for high magnification of individual endplates. YFP and GFP were excited with the 488 nm line of an argon laser and detected using a 522 nm emission filter. Z stacks were flattened with Confocal Assistant and montages of 15 to 40 collapsed image stacks were assembled using Adobe Photoshop. In some cases, the thickness of the muscle caused some lateral movement as the oil immersion objective was focused into deep regions of the muscles. A software program was devised that realigned the images in the stack to remove the lateral drift. In four muscles, each neuromuscular junction from a single motor unit was reconstructed at high magnification (two times zoom, 100$\times$ 1.4 NA objective). Receptor plaque (labeled with rhodamine α-bungarotoxin) and terminal axon area were measured using NIH Image.

In Vivo Time-Lapse Imaging

XFP neonatal mice were anesthetized with an i.p. injection of ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine (Domitor, Orion Corp., Espoo, Finland) cocktail, at a dose of approximately 0.12 mg/pup ketamine and approximately 1.6 μg/pup medetomidine. Anesthetized neonates were intubated and warmed on a temperature-controlled heating element. A superficial incision was made in the ventral neck, and approximately 2% of the acetylcholine receptors in the sternomastoid muscle were then labeled with fluorescently conjugated α-bungarotoxin. Superficial junctions undergoing synapse elimination were imaged at 60$\times$ (water immersion objective, 1.0 NA) using standard epifluorescence microscopy. The animal was then sutured and placed in a heated, oxygenated chamber until sufficiently recovered and active, before returning it to its parents. The animal was allowed to recover for 24 hr before reimaging the same junctions.

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References

Balice-Gordon, R.J., and Lichtman, J.W. (1993). In vivo observations of pre- and postsynaptic changes during the transition from multiple to single innervation at developing neuromuscular junctions. J. Neurosci. *13*, 834–855.

Balice-Gordon, R.J., and Thompson, W.J. (1988). The organization and development of compartmentalized innervation in rat extensor digitorum longus muscle. J. Physiol. *398*, 211–231.

Balice-Gordon, R.J., Chua, C.K., Nelson, C.C., and Lichtman, J.W. (1993). Gradual loss of synaptic cartels precedes axon withdrawal at developing neuromuscular junctions. Neuron *11*, 801–815.

Barber, M.J., and Lichtman, J.W. (1999). Activity-driven synapse elimination leads paradoxically to domination by inactive neurons. J. Neurosci. *19*, 9975–9985.

Bennett, M.R., and Lavidis, N.A. (1984). Development of the topographical projection of motor neurons to a rat muscle accompanies loss of polyneuronal innervation. J. Neurosci. *4*, 2204–2221.

Bennett, M.R., and Pettigrew, A.G. (1974). The formation of synapses in striated muscle during development. J. Physiol. Lond. *241*, 515–545.

Bennett, M.R., McGrath, P.A., Davey, D.F., and Hutchinson, I. (1983). Death of motorneurons during the postnatal loss of polyneuronal innervation of rat muscles. J. Comp. Neurol. *218*, 351–363.

Bernstein, M., and Lichtman, J.W. (1999). Axonal atrophy: the retraction reaction. Curr. Opin. Neurobiol. *9*, 364–370.

Betz, W.J., Caldwell, J.H., and Ribchester, R.R. (1979). The size of motor units during post-natal development of rat lumbrical muscle. J. Physiol. *297*, 463–478.

Bixby, J.L., and VanEssen, D.C. (1979). Regional differences in the timing of synapse elimination in skeletal muscles of the neonatal rabbit. Brain Res. *169*, 275–286.

Brown, M.C., Jansen, J.K.S., and VanEssen, D. (1976). Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. J. Physiol. *261*, 387–422.

Bullock, T.H. (2000). Revisiting the concept of identifiable neurons. Brain Behav. Evol. *55*, 236–240.

Caroni, P. (1997). Overexpression of growth-associated proteins in the neurons of adult transgenic mice. J. Neurosci. Methods *71*, 3–9.

Chen, C., and Regehr, W.G. (2000). Developmental remodeling of the retinogeniculate synapse. Neuron *28*, 955–966.

Colman, H., Nabekura, J., and Lichtman, J.W. (1997). Alterations in synaptic strength preceding axon withdrawal. Science *275*, 356–361.

Edström, L., and Kugelberg, E. (1968). Histochemical composition, distribution of fibres and fatiguability of single motor units. J. Neurol. Neurosurg. Psychiatry *31*, 424–433.

Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., Gross, J., Nerbonne, J.M., Lichtman, J.W., and Sanes, J.R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron *28*, 41–51.

Fladby, T., and Jansen, J.K. (1987). Postnatal loss of synaptic terminals in the partially denervated mouse soleus muscle. Acta. Physiol. Scand. *129*, 239–246.

Fladby, T., and Jansen, J.K.S. (1988). Selective innervation of neonatal fast and slow muscle fibers before net loss of synaptic terminals in the mouse soleus muscle. Acta. Physiol. Scand. *134*, 561–562.

Gan, W.-B., and Lichtman, J.W. (1998). Synaptic segregation at the developing neuromuscular junction. Science *282*, 1508–1511.

Gan, W., Bishop, D.L., Turney, S.G., and Lichtman, J.W. (1999). Vital imaging and ultrastructural analysis of individual axon terminals labeled by iontophoretic application of lipophilic dye. J. Neurosci. Meth. *93*, 13–20.

Gatesy, S.M., and English, A.W. (1993). Evidence for compartmental identity in the development of the rat lateral gastrocnemius muscle. Dev. Dyn. *196*, 174–182.

Jansen, J.K.S., and Fladby, T. (1990). The perinatal reorganization of the innervation of skeletal muscle in mammals. Prog. Neurobiol. *34*, 39–90.

Keller-Peck, C.R., Feng, G., Sanes, J.R., Yan, Q., Lichtman, J.W., Snider, W.D. (2001). Glial cell line derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. J. Neurosci. *21*, 6136–6146.

Kopp, D.M., Perkel, D.J., and Balice-Gordon, R.J. (2000). Disparity in neurotransmitter release probability among competing inputs during neuromuscular synapse elimination. J. Neurosci. *20*, 8771–8779.

Laskowski, M.B., Colman, H., Nelson, C., and Lichtman, J.W. (1998). Synaptic competition during the reformation of a neuromuscular map. J. Neurosci. *18*, 7328–7335.

Lichtman, J.W., and Colman, H. (2000). Synapse elimination and indelible memory. Neuron *25*, 269–278.

Lohof, A.M., Delhaye-Bouchaud, N., and Mariani, J. (1996). Synapse elimination in the central nervous system: functional significance and cellular mechanisms. Rev. Neurosci. *7*, 85–101.

Nguyen, Q.T., Parsadanian, A.S., Snider, W.D., and Lichtman, J.W. (1998). Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science *279*, 1725–1729.

Ontell, M., and Kozeka, K. (1984a). The organogenesis of murine striated muscle: a cytoarchitectural study. Am. J. Anat. *171*, 133–148.

Ontell, M., and Kozeka, K. (1984b). Organogenesis of the mouse extensor digitorum longus muscle: a quantitative study. Am. J. Anat. *171*, 149–161.

Purves, D., and Lichtman, J.W. (1980). Elimination of synapses in the developing nervous system. Science *210*, 153–157.

Sanes, J.R., and Lichtman, J.W. (1999). Development of the vertebrate neuromuscular junction. Annu. Rev. Neurosci. *22*, 389–442.

Thompson, W., and Jansen, J.K.S. (1977). The extent of sprouting of remaining motor units in partly denervated immature and adult rat soleus muscle. Neuroscience *2*, 523–535.

Thompson, W.J., Sutton, L.A., and Riley, D.A. (1984). Fibre type composition of single motor units during synapse elimination in neonatal rat soleus muscle. Nature *309*, 709–711.

Vidal, M., Morris, R., Grosveld, F., and Spanopoulou, E. (1990). Tissue-specific control elements of the Thy-1 gene. EMBO J. *9*, 833–840.