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Pervasive Synaptic Branch Removal in the Mammalian Neuromuscular System at Birth

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SUMMARY

Using light and serial electron microscopy, we show profound refinements in motor axonal branching and synaptic connectivity before and after birth. Embryonic axons become maximally connected just before birth when they innervate ~10-fold more muscle fibers than in maturity. In some developing muscles, axons innervate almost every muscle fiber. At birth, each neuromuscular junction is coinnervated by approximately ten highly intermingled axons (versus one in adults). Extensive die off of terminal branches occurs during the first several postnatal days, leading to much sparser arbors that still span the same territory. Despite the extensive pruning, total axoplasm per neuron increases as axons elongate, thicken, and add more synaptic release sites on their remaining targets. Motor axons therefore initially establish weak connections with nearly all available postsynaptic targets but, beginning at birth, massively redistribute synaptic resources, concentrating many more synaptic sites on many fewer muscle fibers. Analogous changes in connectivity may occur in the CNS.

INTRODUCTION

Despite the widespread belief that neural circuit formation is the central theme of vertebrate neural development, there is ample evidence of the opposite: postsynaptic target cells in various parts of the central and peripheral nervous system appear to be innervated by more axons early in postnatal life than later on (Purves and Lichtman, 1980). The reduction in the number of converging axons, known as synapse elimination, may play a role in establishing permanent synaptic circuits based on experience (Lichtman and Colman, 2000). In the neuromuscular system, this phenomenon has been studied by us and others, especially during the second postnatal week in rodents when muscle fibers make the transition from double and occasionally triple innervation to their adult state of single innervation (Sanes and Lichtman, 1999; Tapia and Lichtman, 2013). For technical reasons, it has remained unclear whether much more extensive circuit alterations occur in the first postnatal week or even prenatally. Knowing the extent of the early developmental reorganization would be helpful in resolving several outstanding questions. For example, in mature muscles, motor neurons tend to innervate muscle fibers of a single type. The origin of this so-called motor unit homogeneity remains incompletely understood, with a number of different factors putatively playing a role including the following: specific targeting of axons to certain muscle fibers and not others, conversion of axons by retrograde signals from the muscle fibers, conversion of muscle fibers by activity or other signals from nerves, and synapse elimination of mismatched nerve-muscle connections. Knowing which axons initially contact each muscle fiber would be helpful in understanding the importance of several of these possibilities. Moreover, study of the developing neuromuscular system can reveal detailed circuit information, such as the number of postsynaptic cells innervated by an axon or the contact areas of all the different axons innervating the same postsynaptic cell, data that would be difficult to obtain in less accessible parts of the nervous system. Given that analogous developmental reorganizations appear to occur in many other parts of the nervous system, this neuromuscular data may provide insights that are useful for a general understanding of neural circuit maturation. We were also motivated to study early synaptic rearrangement because of uncertainty about its role in circuit development. In particular, we were interested to know whether early synaptic rearrangements are ostensibly minor refinements that "functionally validate" or "error correct" connectivity patterns (Cowan et al., 1984; Jacobson, 1969) or perhaps have a more central role of specifying the connectivity.

In this work, we use techniques that give direct measures both of the size of motor units (divergence) and the number of axons that innervate each muscle fiber (convergence). Our results show that at birth, axons transiently project to nearly an order of magnitude more muscle fibers than later and that each

neuromuscular junction is innervated by roughly 10-fold more axons. The many extra axonal branches originate from the same neurons that provide the few branches that ultimately survive development and are spatially intermingled with the surviving branches. Thus, it is likely that local interactions at each postsynaptic target cell, such as those mediated by activity-dependent synaptic competition, not only underlie the final stages of minor refinement in the second postnatal week in mice but also the massive early loss of synaptic connections beginning just before birth.

RESULTS

Motor Units in Young Animals: Massive Divergence

In order to reconstruct motor axon arbors in fetal and very young animals, we used "YFP-H" mice that we had previously found expressed cytoplasmic yellow fluorescent protein (YFP) in very small numbers of motor axons (Feng et al., 2000). Because of the developmental regulation of the promoter used in these transgenic animals (from the *thy1* gene), our previous studies detected very faint or no fluorescence in these and other subset-expressing lines prior to postnatal day (P) 7 (Keller-Peck et al., 2001). However, when we amplified the signal by fluorescent immunohistochemistry, we could clearly detect YFP-expressing axons in very young animals (Figure 1), albeit rarely. We surveyed ~4,000 neck muscles (the sternomastoid, cleidomastoid, and clavotrapezius) between embryonic day (E) 16 and P4 and found 23 in which a motor axon arbor was labeled sufficiently well that all of the branches were visible to each terminal. We discarded approximately ten other motor axons in which the labeling was deficient or in which inadvertent damage to the muscle precluded quantifying the full complement of branches. The 23 well-labeled motor units were reconstructed by stitching together confocal image stacks obtained at the diffraction limit using high numerical aperture (NA) oil objectives. Sites of synaptic contact were assessed by three-dimensional colocalization of a terminal branch and the postsynaptic plaque of acetylcholine receptors (AChRs) labeled with fluorescently tagged alpha bungarotoxin (see Experimental Procedures for details).

Imaging the neonatal motor units at high resolution showed that at birth, each axonal contact to a muscle fiber emanated from a single branch of a motor axon that could be traced to a proximal bifurcation in the axonal arbor (Figures 1A–1D), as is the case in more mature neuromuscular junctions (Figure 1I). However, in many other ways, the axonal innervation of muscles fibers was different.

Smaller-Caliber Branches

First, the caliber of axons was significantly smaller when compared to motor axons in older mice (Figure 1F). On average, in the perinatal period, the main branch of the axons that entered the muscle had a diameter of 1.48 ± 0.03 μm (n = 40 measurements from 10 motor units) compared to 4.08 ± 0.07 μm (n = 48 measurements from 12 motor units) at 2 weeks of age ($p \leq 0.0001$, Student's t test). The terminal branches of perinatal motor axons were even finer, and many were measured to be at the diffraction limit of the imaging objective and thus ≤0.22 μm in diameter (NA = 1.4, Alexa 488 emission at 515 nm).

Giant Motor Units

A second difference was that axons from the perinatal period were much more branched when compared to the sparse branching found in animals older than 2 weeks of age (compare Figures 1H to 1I). For the most part, the extra branching in perinatal motor units did not generate blind ends. Rather, as was the case in older animals, >99% of nerve terminal branches terminated on AChR-rich postsynaptic sites. For example, whereas in the cleidomastoid each motor axon in 2-week-old mice innervated, on average, 18.8 ± 3.0 (n = 5) muscle fibers, each neonatal axon had terminal contacts with the receptor-rich regions on 221 ± 6.1 (n = 5) different muscle fibers, a highly significant 11.8-fold ± 2.2-fold change in size (compare Figures 1E and to 1G, light gray ovals represent the AChR sites, yellow plaques represent AChR sites innervated by the labeled motor unit; $p < 0.001$, Student's t test). A similar order of magnitude difference in motor unit size relative to motor units in adults was also present in the two other ventral neck muscles studied (sternomastoid and clavotrapezius) (Table 1). However, in contrast to the change in the size of motor units, the total number of neuromuscular junction sites containing AChRs (labeled with fluorescently tagged alpha bungarotoxin) remained stable from E18 onward (also see below). In the cleidomastoid, for example, there were 410 ± 23 (n = 5) neuromuscular junctions at birth (one per muscle fiber), as compared to 413 ± 13 (n = 5) 2 weeks later (not significantly different [p = 0.898]; Student's t test). Thus, the greater number of synaptic branches in the perinatal period must be distributed over the same limited number of neuromuscular junctions, demonstrating that each motor axon innervates a 10-fold greater proportion of muscle fibers at birth than 2 weeks later.

Small Synapses

A third difference between perinatal and older axons was the size and postsynaptic coverage of individual synaptic terminals. In contrast to ~100% occupation of neuromuscular junction AChR sites by single axons in adults, each terminal axon branch at birth typically occupied only a small portion of a neuromuscular junction's AChR territory (Figures 1A–1D). In the cleidomastoid muscle in E18–P0 animals, each labeled terminal axonal branch covered, on average, 14.2% (±11.4%, n = 151) of the total AChR area per contacted junction. This small percentage of occupation probably overestimates the actual area of synaptic contact, because it includes nonsynaptic connector branches (see electron microscopy section below). Even so, of the 151 junctions studied, only one was innervated by an axon that overlapped with more than 50% of the junctional area (Figure 1J). The typically small contact area of single axonal input to neuromuscular junctions suggests that each developing neuromuscular junction may be shared by many different axons. Indeed, when we looked at neonatal neuromuscular junctions in a transgenic fluorescent protein-expressing mouse line that labels all motor axons ("YFP-16"; Feng et al., 2000), we saw that the cumulative synaptic drive to each neonatal neuromuscular junction was much greater than that shown by single axon labeling (compare Figures 1A–1D with 1K). With all axons labeled, each perinatal junction was nearly fully occupied (92.4% ± 5.0%, n = 33, of the receptor area covered; Figure 1K). The synaptic vesicle marker synaptophysin was also present throughout each

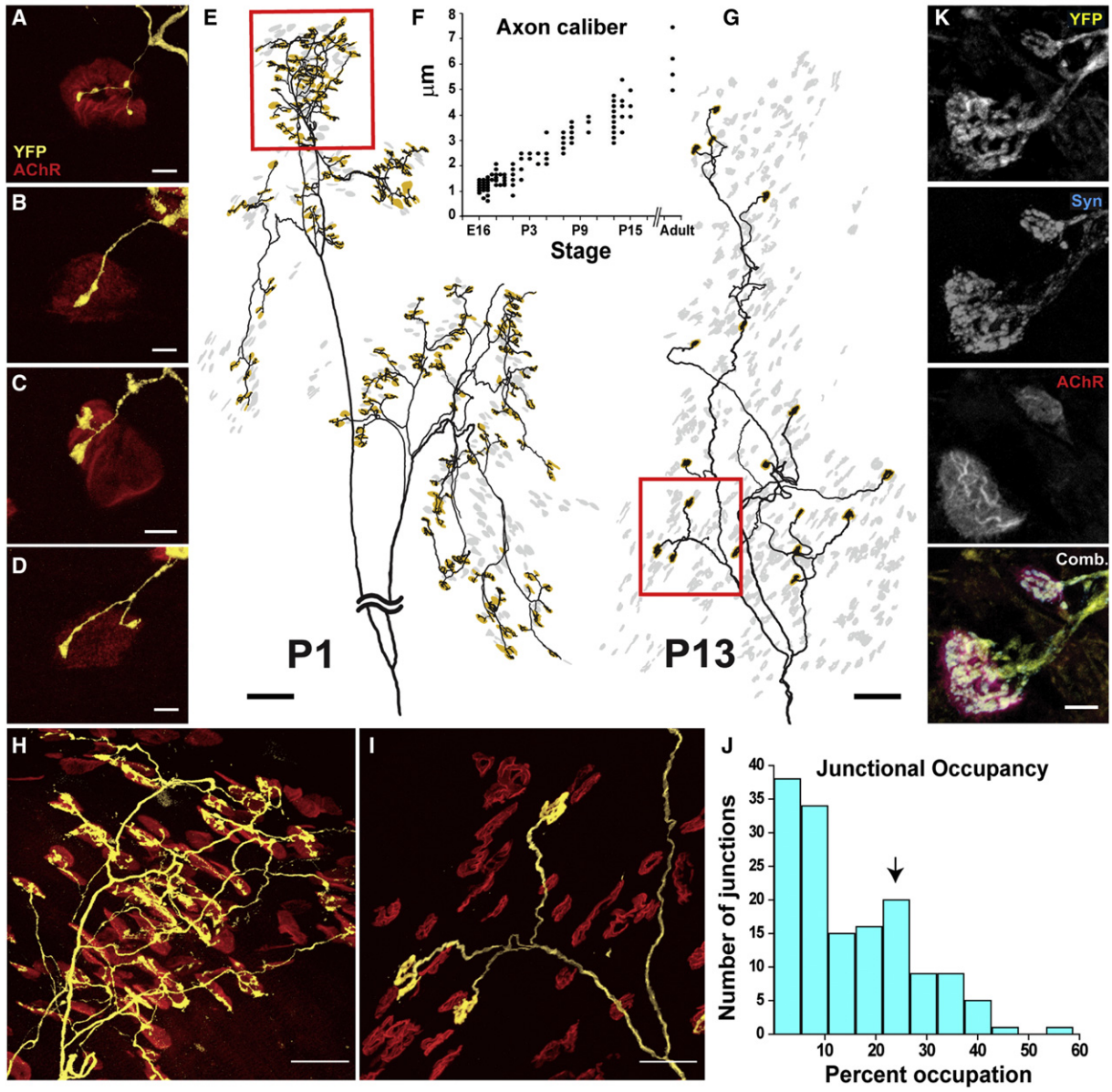

Figure 1. Comparison of Motor Units at Birth and 13 Days Later Shows Profound Changes in Axonal Arbors and Synapses

(A–D) High-resolution confocal images of four individual neuromuscular junctions from P1 showing axons (yellow) occupying small regions of AChR sites (red).

(E) Montage of a motor unit at P1 in the cleidomastoid muscle showing a massive number of neuromuscular junctions innervated (yellow) by the immature motor axon. Noncontacted junctions are indicated in gray.

(F) Shows a graph with the changes in motor axon caliber over development. Four measurements 5 μm apart were taken for each of the 23 labeled axons from birth. The later-stage axons were also taken from YFP-H animals.

(G) Montage of a single motor unit at P13 in the same muscle. The arbor is shown (black) along with the rather sparse number of neuromuscular junctions innervated by the labeled axon (yellow) and the uncontacted junctions (gray).

(H) Micrograph of the confocal image stacks from the boxed region in (E), showing the axon labeled with YFP (yellow) and the postsynaptic receptors labeled with Alexa 594-conjugated α-bungarotoxin (red).

(I) Micrograph of boxed region in (G).

(J) The graph shows the distribution of synaptic territory occupied by motor nerve terminals at birth. Data were obtained from 151 neuromuscular junctions (NMJs). Arrow indicates the average terminal occupancy.

(K) High-resolution confocal image of two perinatal junctions showing the presynaptic terminals (all axons labeled, yellow), synaptophysin labeling (blue), and receptor staining (red). Scale bars represent 5 μm in (A)–(D) and (K), 100 μm in (E) and (G), and 30 μm in (H) and (I).

Table 1. Motor Unit Data

Muscle	Age	Motor Unit Size		Percentage of NMJs Innervated
Sternomastoid*		Actual	(Norm.)	
	E16	22	(0.94)	18.2%
	E16	58	(2.47)	35.7%
	E16.5	24	(1.02)	28.0%
	E16.5	62	(2.64)	49.0%
	E17	47	(2.01)	48.8%
	E17	197	(8.40)	61.9%
	E17	99	(4.22)	61.4%
	E18	200	(8.53)	65.4%
	P0	157	(6.70)	70.0%
	P1	160	(6.83)	80.4%
	P1	115	(4.91)	64.0%
	P2	285	(12.16)	60.2%
	P3	179	(7.64)	37.1%
	P3	158	(6.74)	32.8%
	P5	118	(5.03)	25.7%
	P7	73	(3.11)	13.1%
	P7	54	(2.30)	12.1%
	P7	45	(1.92)	9.2%
	P8	56	(2.39)	11.7%
	P8	68	(2.90)	10.3%
	P10	51	(2.18)	8.3%
	P13	37	(1.58)	8.7%
	P13	31	(1.32)	7.3%
	P13	17	(0.73)	6.8%
	P13	14	(0.60)	4.6%
	P13	16	(0.68)	3.1%
	P14	33	(1.51)	5.8%
	P15	19	(0.81)	6.2%
	P21	27	(1.15)	8.9%
	P21	17	(0.73)	4.7%
Cleidomastoid				
	E16	52	(2.77)	32.3%
	E17.5	219	(11.65)	50.8%
	E18	228	(12.13)	50.9%
	E18	198	(10.53)	59.5%
	P0	231	(12.29)	59.8%
	P0	229	(12.18)	50.8%
	P1	276	(14.68)	49.7%
	P4	31	(1.65)	12.9%
	P5	68	(3.62)	16.6%
	P7	29	(1.54)	7.1%
	P8	30	(1.60)	7.1%
	P13	18	(0.96)	4.4%
	P13	18	(0.96)	4.2%
	P13	15	(0.80)	3.8%
	P13	13	(0.69)	3.4%
	P14	30	(1.60)	6.6%
Clavotrapezius				
	E18	331	(13.97)	80.3%
	P23**	24	(1.00)	4.6%
	P23**	24	(1.00)	4.5%

*Given the compartmentalized nature of the sternomastoid, it was meaningless to calculate percent occupation of NMJs as a fraction of the entire muscle. Thus, we computed this value for the cohort of NMJs in the region to which the axon projected. Areas where the compartment was unambiguously isolated were analyzed and the overall average was reported.

**Single axon expression was scarce in the clavotrapezius. Thus, these adult values were computed using two muscles with multiple axons labeled (nine in each) at an age in which each NMJ has one input. Thus, the adult size is reported as the total number of labeled contacts divided by the number of labeled axons.

junction (Figure 1K), arguing that the majority of these contacts are synaptic. However, the small size of perinatal neuromuscular junctions compounded by the tight fasciculation of the incoming axons and their small caliber made it impossible to directly assess the number of converging axons at neonatal junctions by fluorescence microscopy given the limitations imposed by diffraction (see below).

Peak Motor Unit Size Just before Birth

To learn when axonal arbors projected to the greatest number of muscle fibers, we also screened embryonic muscles from YFP-H and GFP-S mice for ones that contained a single fluorescent motor axon. Analysis of motor neuron axon arbors from embryonic periods (E16–E18) showed that the size of motor units increased over prenatal life to reach a peak just before birth. We found that at E18 (1 day before birth), motor units are larger than the first day after birth. An example of this change is presented in Figure 2A, which shows a clavotrapezius motor unit at E18 whose arbor extends to 331/412 muscle fibers. This axon projects to 80.3% of the neuromuscular junctions, whereas the average axonal projection was 4.6% of the muscle fibers in P23 animals (Table 1). However, 3 days before birth (E16), motor unit sizes were, on average, ~6-fold smaller than at E18 (n = 5; see Table 1). Figure 2B shows an axon reconstructed from an E16 cleidomastoid muscle in which the labeled axon innervates 52 of 161 (32.3%) of the total number of neuromuscular junction sites. Part of the change in motor unit size between E16 and E18 was related to an increase in the number of muscle fibers within the muscle because the E16 motor units projected to muscles that apparently were still adding muscle fibers. For example, in five E16 cleidomastoid muscles, there were 2.5-fold ± 0.2-fold fewer AChR-containing postsynaptic sites than in adults (E16: 165.5 ± 5.0 [n = 4] versus adult: 413 ± 13.0 [n = 5]). Secondary myogenesis is complete by birth because the number of postsynaptic receptor sites reaches its adult level by then (see above). The mismatch between the increases in the number of postsynaptic sites added (2.5-fold) in late embryos and the larger increase in the size of motor units (4.3-fold) means that many of the newly added axonal branches do not project exclusively to

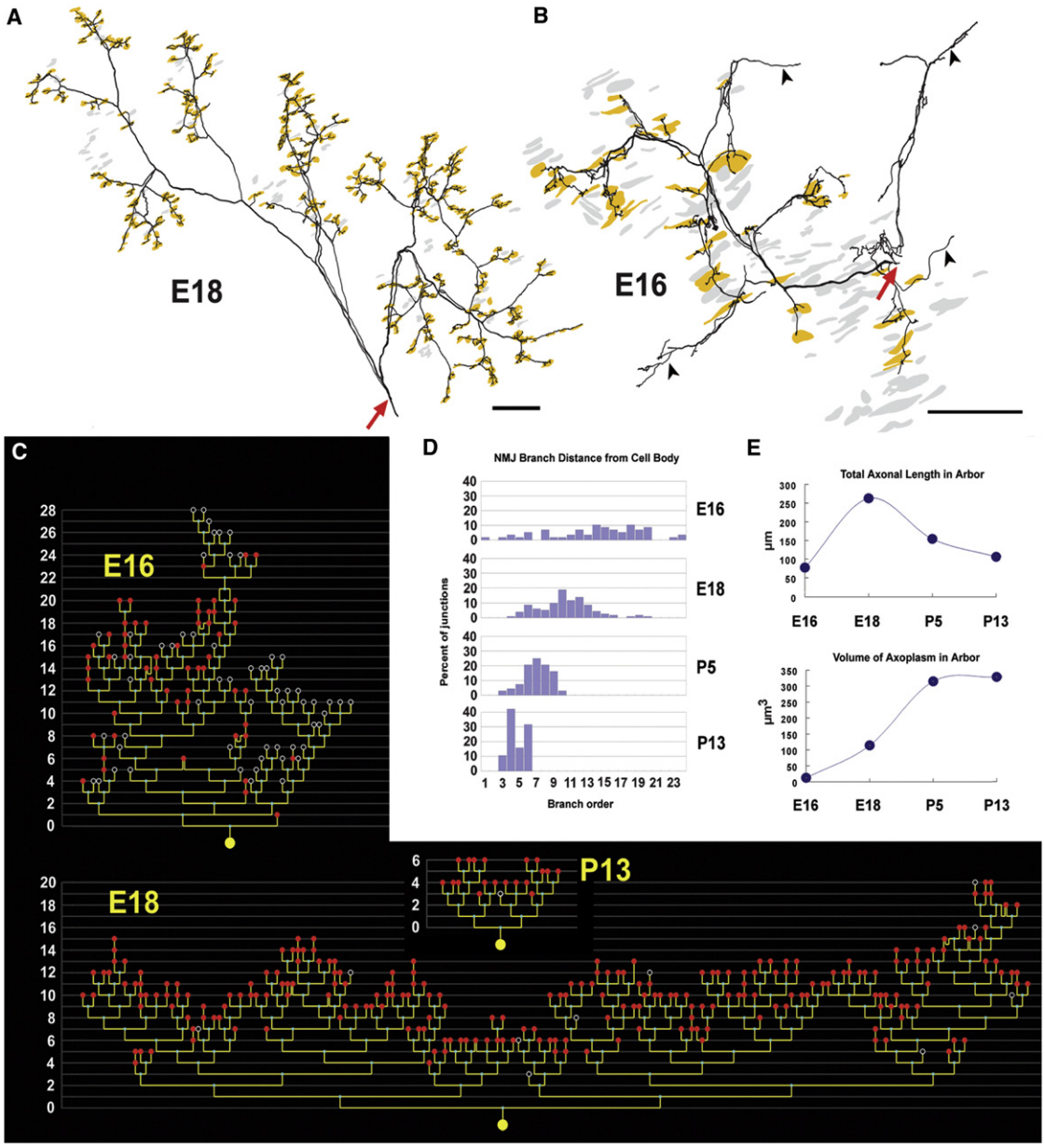

Figure 2. Embryonic Motor Unit Reconstructions and Analysis

(A) Montage of a single motor unit at E18. Red arrow indicates main entering branch of axon, yellow plaques are innervated junctions, and gray plaques are uncontacted junctions.

(B) Montage of a single motor unit at E16 showing escaped fibers (arrowheads). Red arrow shows entry point of axon in (A) and (B).

(C) Schematic branching diagram of three examples of motor units at E16, E18, and P13 (mature), respectively. Branch order indicates the number of branches between the terminal and the axon entry point to the muscle. Axons are shown (yellow) along with contacted junctions (red circles), endings which do not terminate on receptor clusters (hollow white circles), and branch points (blue dots).

(D) Graph showing the number of terminal endings in these examples having a given branch order as a function of age.

(E) Graphs showing the total length of branches and the volume of axoplasm of representative arbors over development. Scale bars represent 50 μm in (A) and (B).

the newly added muscle fibers. Thus, in mice, neuromuscular wiring complexity (i.e., motor unit size) peaks just before birth and rapidly simplifies over the first several postnatal days (see Figure 3C).

In addition to the branches that contacted muscle fibers, the embryonic motor axons also possessed numerous branches that did not terminate at AChR sites, something that was extremely rare at later stages (arrowheads, Figure 2B). Some of these branches wandered quite far from the band of neuromuscular junctions, as has previously been observed in embryonic muscles (see, for example, Lupa and Hall, 1989). Given that motor units are still enlarging as new fibers are being added

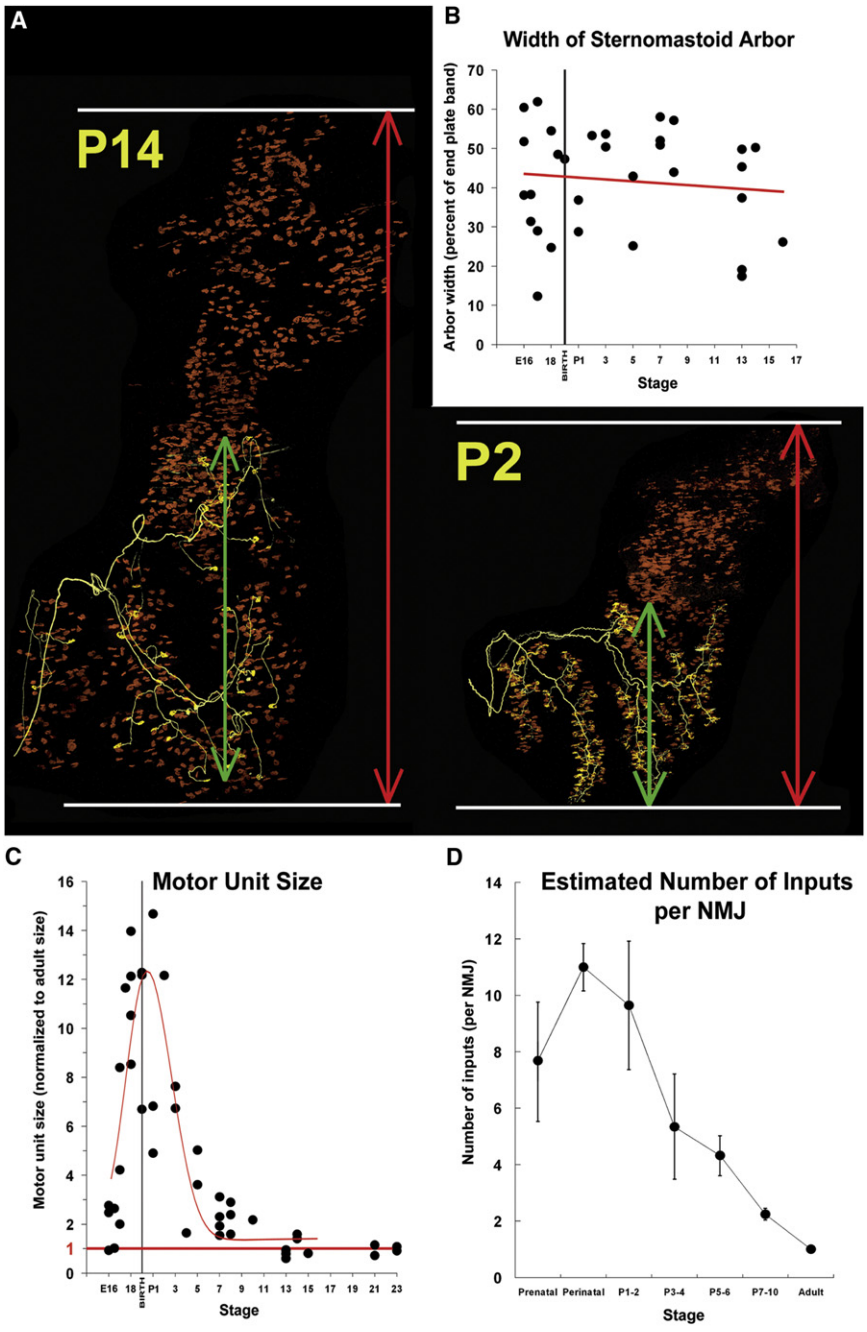

Figure 3. Effects of Branch Trimming on Motor Unit Size and Shape during Development

(A) Confocal image montages showing how arbor width was measured at both P14 and P2. The red arrow indicates the endplate band width, while the green arrow is the arbor width in the same axis.

(B) Arbor widths in the sternomastoid as a percentage of total endplate band width over development. Red line is a best fit to the data.

(C) Motor unit size as a function of developmental day. Sizes are expressed as a "fold" change from adult size—that is, a size of 12 means a motor arbor contacts 12 times the number of postsynaptic cells than the average adult arbor in the same muscle. Red curve is a best Gaussian fit to the data.

(D) Graph showing the estimated average number of inputs to each neuromuscular junction over development based on motor unit size. Error bars represent mean ± SEM.

in embryonic life, it is possible that these nerve sprouts serve the purpose of surveying the muscle for new synaptic sites.

Pruning Predominately by Terminal Branch Loss

Because there are several different ways an axon might prune its branches (e.g., by lopping off major proximal limbs with many synaptic branches lost at once versus more piecemeal pruning of individual terminal branches), we constructed full branching diagrams at various ages to decide how the branch loss occurred (Figure 2C). Analysis of the branching trees showed that at all early developmental ages, axons began branching shortly after entering the muscle, with most of the initial branches giving rise to more branches and multiple synapses on each branch limb. Thus, the majority of terminal divisions occur only after a number of initial relatively symmetric branching occurrences. This style of branching is similar to the ramification pattern seen in later development and in adults (Keller-Peck et al., 2001; Lu et al., 2009). We calculated the branch order for each terminal (i.e., synaptic) branch in an axonal arbor by counting the number of branch points between a neuromuscular junction and the axon entry site to the muscle. The mean branch order for motor axons decreased progressively with age, dropping from 11 to 4 between E18 and P13 (Figure 2D). This large decrease is more consistent with what would happen with loss of many individual distal terminal branches, as opposed to what would happen if a more proximal multisynaptic branch were pruned. Even if a large proximal branch of an axon that eliminated half of an axon's arbor were lost, the effect would be to reduce the branch order by only one—far less than the branch order actually drops. This conclusion was also corroborated by experiments mentioned below.

Total Axoplasm per Neuron Increases as Branches Are Pruned

Because a mouse's muscles and skeleton are growing at the time branches are being removed, it is possible that, despite the loss of a large number of branches, there is no net loss of axonal material supported by each motor neuron soma. In particular, no net loss might occur if the remaining branches had to elongate to keep pace with the growth of the muscle.

To explore this issue, we calculated the length, surface area, and volume of axonal motor units within the sternomastoid muscle at various developmental ages. Our measurements showed that branch pruning did cause the total length of an axon's branches within the muscle to decrease between birth and 13 days postnatally. However, the total amount of axoplasm in these arbors actually increased (Figure 2E; see Experimental Procedures for details of analysis). The net increase in axonal material was due to an increase in both the length within the muscle of the remaining axon branches and an increase in their calibers. Thus, despite the profound branch loss, a motor neuron's total axoplasmic volume once the axon reaches the target muscle is actually increasing over this developmental period. Given that the distance between the muscle target and the neuronal cell body is also increasing due to animal growth, the total increase in axoplasm per neuron is even greater than what we have measured.

Overall Distribution of Axonal Targets Is Unchanged by Pruning

One potential reason for the pruning is that it restricts the spatial extent of an axonal arbor to focus an initially diffuse projection into a more circumscribed area. In the small clavotrapezius and cleidomastoid muscles, nearly all adult motor units extend throughout the entire muscle, so spatial focusing cannot be occurring in these muscles (Lu et al., 2009). We could analyze the possibility of spatial refinement of motor axons in the sternomastoid muscle because in maturity, each motor axon was confined to a small subregion of the muscle (Figure 3A) (see also Keller-Peck et al., 2001). We found that relative to the area of the muscle, there was no significant change in the extent of motor arbors between the young ages and later (compare Figures 3A and 3B). The fact that motor axon arbors do not become more limited in extent implies that the impetus for branch removal at early stages is not based on the position of the branch within the muscle. This result is also consistent with the data mentioned above arguing against proximal branch trimming, because each proximal branch typically projects to nonoverlapping regions of the muscle's endplate band (see also Lu et al., 2009); therefore loss of a proximal branch would have been expected to focus an axon's projection to a smaller territory.

Neuromuscular Junctions in Young Animals: Massive Convergence

The results already described indicate that axons innervate more postsynaptic target cells at birth than later. Given the fixed number of postsynaptic sites and assuming no change in the number of innervating axons projecting to a muscle, these results imply that there could be as many as 11 axons converging at each neuromuscular junction at birth (Figure 3D). The limited occupancy of the postsynaptic site by individual axons (see, for example, Figures 1A–1D and 1J) further supports this idea because at most neuromuscular junctions at birth, there is certainly room for many axons to establish synapses. But this estimate assumes that there is no dominant axon at each junction that occupies a large percentage of the territory, and our calculation is also based on the assumption that the number of innervating axons projecting to the muscle remains constant. We therefore needed to obtain a more direct measure of the number axons converging at neuromuscular junctions at birth. We wanted in addition to assay each of these contacts in terms of its size. Thin-section serial scanning electron microscopy of perinatal neuromuscular junctions provided this information (see Experimental Procedures). Seven hundred serial sections (30 nm in thickness) were imaged in the region of the endplate band and three neuromuscular junctions on adjacent muscle fibers were completely reconstructed (Figure 4A, top panel). Because, as already mentioned, single motor unit labeling showed that axons sent only one branch to each junction they innervated (see Figures 1A–1D), it was possible to count the number of different axons converging at the junction by looking at the number of axons entering the junctional site. We counted 7, 8, and 11 axons entering the three adjacent junctions (Figure 4 and see Figure S1D available online). In each case, all the axons were bundled in a single fascicle and entered the junctional site from the same direction. All (26/26) of the axons entering the junctions were unmyelinated, although a few myelinated motor or sensory axons were visible in the nerve fascicles coursing through the muscle.

At Birth, Most Terminal Branches Establish Neuromuscular Synapses, but a Few Appear to Be Recently Eliminated

To quantify how many of the converging axons were actually establishing synaptic contact with the underlying muscle fiber, we identified all the sites where vesicle-filled profiles of axons were juxtaposed with the muscle fiber membrane with no intervening glial cell or an open gap of greater than 1 μm. In these three reconstructed junctions, 23/26 (~88%) of the axons had sites of contact with muscle fiber membrane (Figure 4A, bottom). The individual terminal arbors of each of the 11 axons innervating one of these junctions are shown in Figure 4B. The three axons that did not have contact with muscle fibers (see, for example, axons 10 and 11 in Figure 4B) terminated in vesicle- and mitochondria-filled bulbs emerging from quite thin axonal branches. Each of the axons that did not contact the muscle fiber was in close proximity to sheathing Schwann cells that contained axosomes (Figure S1C; the yellow-tinted Schwann cell is also shown in panels (ii) and (iv) in Figure 4C). All of these histological signs suggest that these axons that were near junctions but not innervating them had previously been in contact with the muscle fiber and now were in the process of being eliminated (Bishop et al., 2004; Riley, 1981). Thus, synapse elimination seems to be underway just as animals are being born.

Because of the large volume being reconstructed, it was possible in some cases to trace the axons back far enough to assess whether the same axon was innervating more than one of the three adjacent neuromuscular junctions. Of the 26 terminal axon branches innervating these three junctions, seven were traceable back to branch points where they bifurcated to give rise to innervation to two of the three junctions (Figure 5). In six of the seven cases, the axons innervated comparably sized percentages of each of the junctions (6% versus 10%; 16% versus 10%; 8% versus 17%; 4% versus 10%; 17% versus 14%; 21% versus 16%). In one case, however, we saw that one of the axon branches did not establish a synaptic contact with the neuromuscular junction site but rather terminated in a bulb just proximal to one of the junctions. The ultrastructural

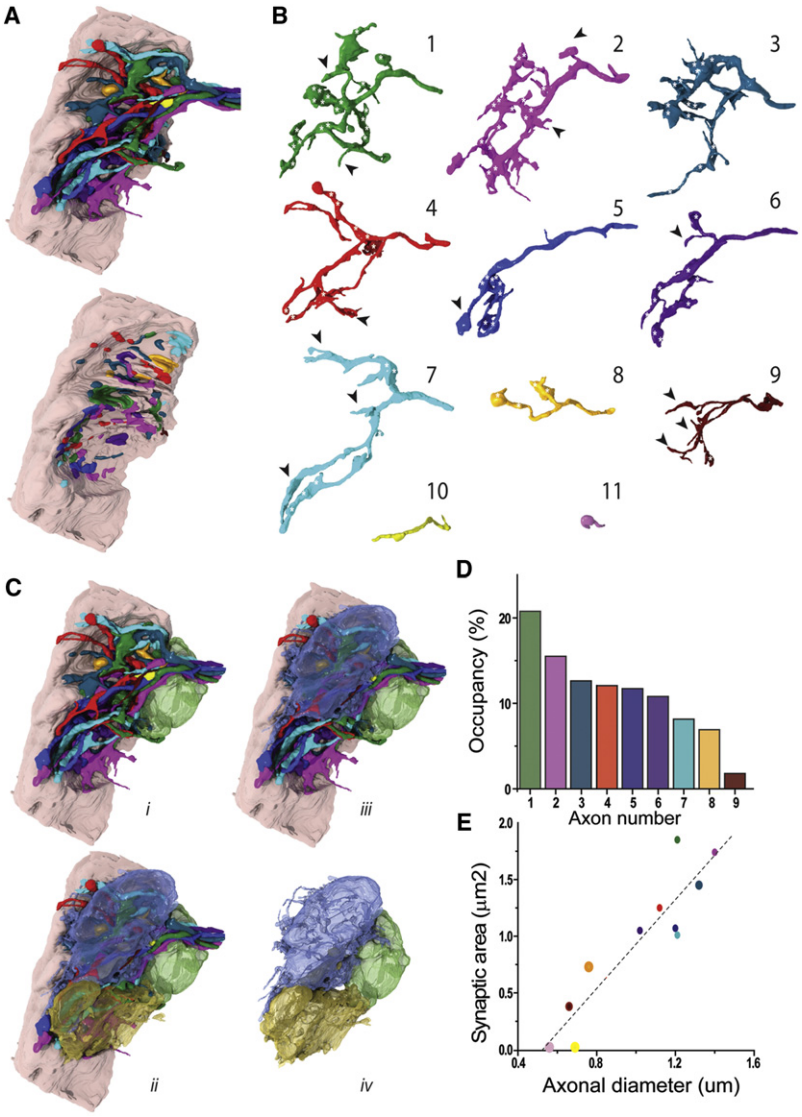

Figure 4. Serial Section Electron Microscopy Showing Multiple Axons Converging at a Neuromuscular Junction in a P0 Mouse Sternomastoid Muscle

(A) Top: shows a three-dimensional surface rendering of a serially reconstructed neuromuscular junction. The colored processes represent each of the axons converging onto the neuromuscular junction site of the muscle fiber (light pink). The 11 axons all enter in a fasciculated bundle from one direction. Several of the axons, however, have small sprouts extending beyond the muscle fiber and are typically not in contact with the fiber beyond the neuromuscular junction region. Bottom: sites of synaptic contact between the axons and the muscle fiber highlighted in the color of each axon. Substantial intermixing of the synaptic territories by different axons is observed.

(B) Renderings of each of the axons innervating (axons 1–9) or in close proximity but not making synapses with (axons 10 and 11) the target cell in numerical size order. The asterisks highlight the synaptic sites associated with each axon. Several axonal branches have no synapses (arrowheads).

(C) Semitransparent surface rendering shows the contiguous areas occupied by the three terminal Schwann cells at this neuromuscular junction (i–iv). The three glial cells each had direct contact with most of the axons when they entered the glial cell's territory, suggesting that the glial cells showed no particular preference for some axons over others.

(D) Size distribution of synaptic contacts for all axons in (B) shows that no axon has more than a minority of the synaptic territory.

(E) Linear relationship between diameter of axons entering the junction and their synaptic occupancy.

appearance of this axonal bulb suggested, as described above, that it was a retracting axon, i.e., there were nearby local shed axosomes, and it had a smooth shape (Bishop et al., 2004), rather than a growth cone (i.e., it showed no filopodia or lamellopodia). This result suggested that an axon branch was already in the process of retracting in the first postnatal day. Another branch of the same axon innervated 15% of the neuromuscular junction area on an adjacent neuromuscular junction (axon 7 in Figure 5). Thus, this early stage of branch loss is occurring asynchronously among the branches of one axon. This result lends further support to the conclusion that the initial axon pruning decisions are being made at the level of terminal branches and not more proximally in the axon arbor. Moreover, the fact that most axons are being maintained at a neuromuscular junction while one is being removed supports the idea that beginning at birth, during the earliest stages of synapse elimination, different axons are being sequentially removed from junctions rather than synchronously.

Extensive Synaptic Intermixing at Birth

The serial reconstructions also provided information about the way multiple axons coinnervated neuromuscular junctions at birth. Many of these features were different from both adult singly innervated neuromuscular junctions and later-stage multiply innervated junctions. The synaptic contacts of the axons were highly intermixed, showing no evidence of the interaxonal segregation found at later stages of the elimination process (Gan and Lichtman, 1998) (Figure 4A). The branches of the different axons were not only intermixed but also were closely juxtaposed to each other, with their membranes abutting without intervening Schwann cell processes (Figure S1A, boxed region). However, as found at older ages, the synapses were associated with a Schwann cell cap (Figure 4C). Even among the branches of one axon, its synaptic regions were distributed extensively over the neuromuscular junction area (Figures 4A, top panel, and 4B, white asterisks). There were also nonsynaptic axonal branches that exited each neuromuscular junction as terminal sprouts. Some of these sprouts headed off the junction by growing out into the extracellular space rather than on the muscle fiber or another cell's membrane (see arrowheads in Figure 4B). Sixteen of 26 axons also had nonsynaptic branches within the junction, something not observed in mature

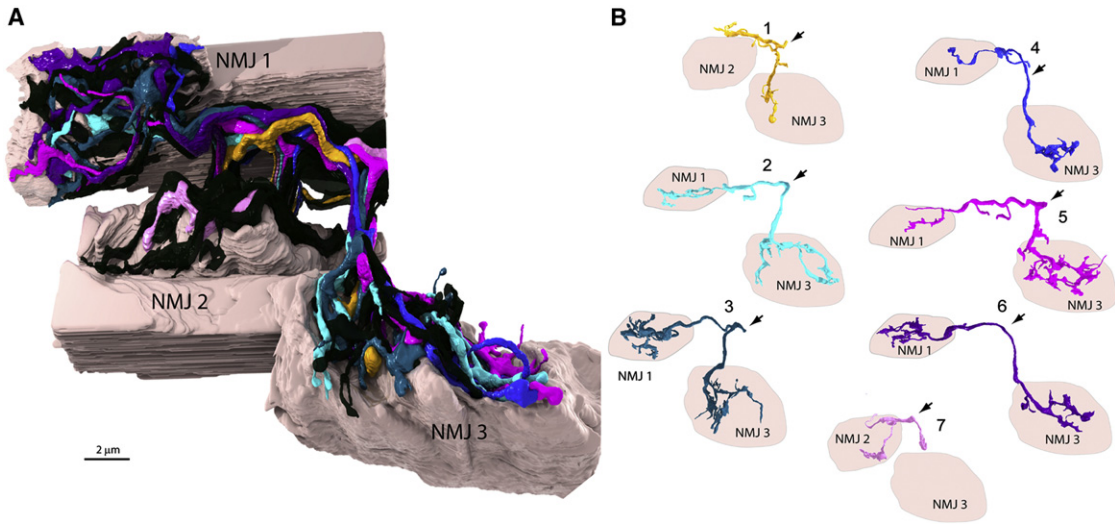

Figure 5. Evidence Showing that Many Axons Project to the Same nearby Neuromuscular Junctions in a Newborn Mouse Sternomastoid Muscle

(A) A three-dimensional reconstruction from serial electron microscopy highlights with colors of seven different motor axons that branch to more than one of three adjacent muscle fiber neuromuscular junctions. The black processes show the rest of the axons.

(B) The territory within each neuromuscular junction (light pink) occupied by these axons is shown. The amount of area occupied by an axon varies between junctions. In one case (axon 7), a terminal branch is retracting from NMJ 3 while the same axon maintains contact with NMJ 2. These data support the idea that the profound changes in neuromuscular connectivity beginning at birth are based on terminal, as opposed to proximal, branch pruning. Arrows indicate the sites of the terminal branch points.

neuromuscular junctions. The vesicle-filled varicosities that abutted the postsynaptic muscle fiber had smaller volumes, a lower density of vesicles on average, and fewer mitochondria than synapses at older junctions (Figure S1A). On the postsynaptic side, there were small shallow folds rather than the typical deeper junctional folds seen at later ages and surprisingly large accumulations of mitochondria in the subsynaptic region of the muscle fiber, which are not so evident in later stages (Figure S1A). Only one or two myonuclei were observed at these neuromuscular junctions compared to three to four at later ages (Bruusgaard et al., 2003).

Glial Cell Territories at Developing Neuromuscular Junctions Do Not Partition between Axons

Given the high degree of intermixing of axon terminals, we were interested to see how glial cells apportioned themselves in these junctions. Might the glial cells at immature neuromuscular junctions associate with some axons more than others and presage the ultimate survivor or soon-to-be-lost inputs? At each of the three reconstructed neuromuscular junctions, there were three terminal Schwann cells. At each junction, these glial cells occupied largely nonoverlapping but contiguous territories, as is the case in older neuromuscular junctions (Brill et al., 2011). Each of these glial cells was in close proximity to the axons innervating the muscle fiber. The Schwann cells at one of the reconstructed junctions are shown in Figure 4C. Small processes emanating from the glia contacted or in some cases completely wrapped parts of the axons (Figure S1A). Despite these interactions, we could find no evidence of Schwann cells favoring some axons (such as those with large or small axonal diameter). In fact, individual glial cells and even individual processes of a glial cell surrounded multiple small and large diameter axons. This ensheathment included axons that appeared to be already disconnected from the muscle fiber. Thus, none of this data supports the idea that Schwann cells are playing a role in either selectively maintaining or selectively weakening axons that are converging on the same neuromuscular junction.

Terminal Axon Caliber Correlates with Synaptic Area

Because only one axon terminal at each neuromuscular junction will ultimately survive the developmental epoch, it was possible that one axon had a different appearance or more dominant foothold on the muscle fiber than the others. However, in none of the three junctions did any axon occupy greater than 30% of the junctional area, consistent with the light microscopy of single axons mentioned above (see Figures 1A–1D). The range in the sizes of the synaptic areas between the various axons seemed to be a continuous distribution with no obvious steps between those with large areas and those with small areas (Figure 4D). Previous work showed that over time, as the dominant axon comes to occupy most of the neuromuscular junction site, it comes to have a larger axon caliber than the axons that are in the process of being eliminated (Keller-Peck et al., 2001; Walsh and Lichtman, 2003). Interestingly, we find here that even at birth, the axons with the most synaptic contact have the largest axonal caliber at the entrance site of the junctions (Figure 4E). Therefore, the axon's caliber at the neuromuscular junction entrance site in newborns is an excellent measure of the area of overlap with AChRs and strongly correlates with the number of contact sites.

The small area of contact of virtually all motor axon inputs (area of contact ranged from 10%–30% of the AChR plaque) suggests that many are too weak to bring the muscle fiber to threshold,

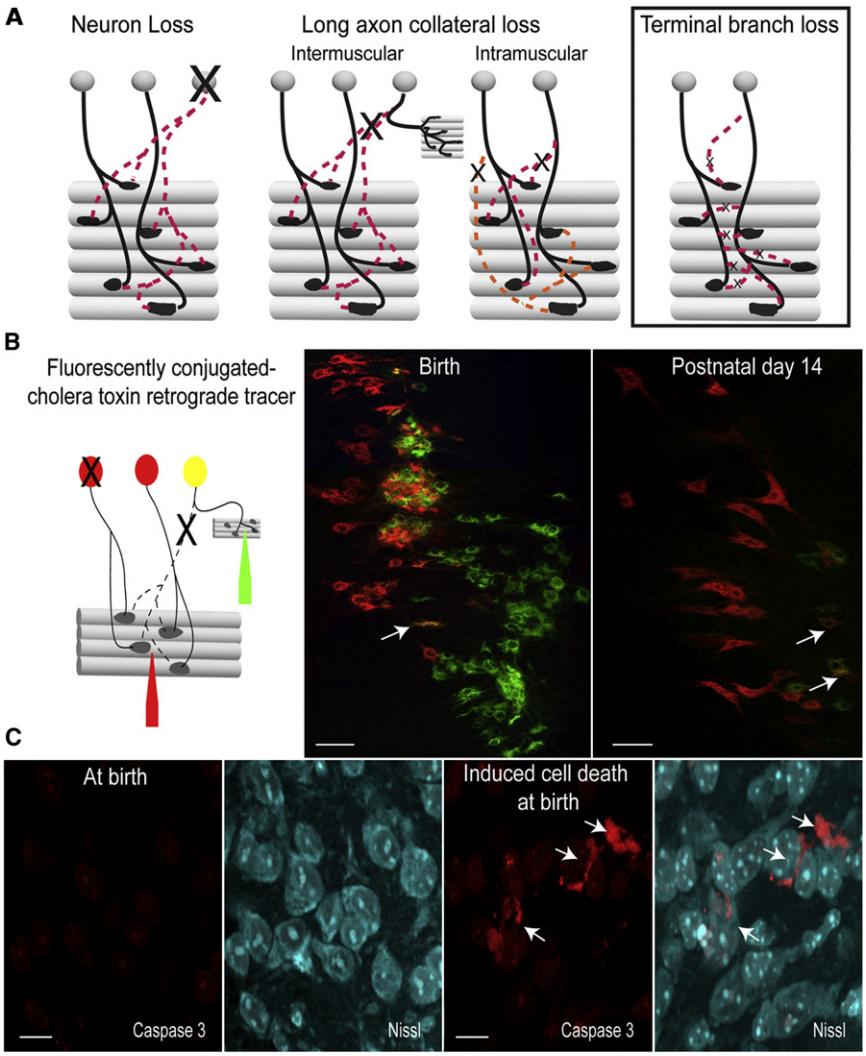

Figure 6. Possible Cellular Mechanisms to Explain Axonal Loss during Early Neuromuscular Development

(A) The diagram indicates four possible scenarios: late motor neuron death (left), loss of long axon collaterals of axons erroneously projecting to more than one muscle (middle, intermuscular), loss of long axon collaterals within a muscle (middle, intramuscular), and pruning of many terminal branches of axons within the target field (right). The evidence presented argues that the boxed alternative (terminal branch loss) is the only one playing a role in the perinatal period.

(B) Diagram showing an experiment testing for transient erroneous projections of axons to multiple muscles in early development (left panel). Two fluorescently labeled cholera toxin fragments (Alexa 488, green and Alexa 594, red) were injected into the nearby cleidomastoid (green pipette) or sternomastoid (red pipette) at P0 and P14 to see whether any doubly labeled neurons were present in the spinal cord. No evidence of early projection mixing was found at P0 (middle panel) nor at P14 (right panel). Specific motor pools for each muscle were clearly visible 24 hr after injection. Although most cells were exclusively green or red labeled, a few faint yellow (double labeled) neurons were observed at P0 (arrow), but the same number was also found at P14 (arrow), suggesting perhaps a small amount of dye spill over between the two nearby muscles. Scale bar represents 100 μm.

(C) No evidence of motor neuron cell death at the time of massive motor axon branch loss after birth. Left: there was no caspase-3 labeling (red) or TUNEL (data not shown) in cervical ventral horn neurons at birth (Nissl staining, blue) within the sternomastoid motor pool (i.e., cervical levels 1–4). Right: cell death, however, could be induced by axotomy of the nerve to the sternomastoid muscle at birth. Cells undergoing apoptosis (caspase 3 labeling, red, arrows) were observed in some of the neurons of the sternomastoid motor pool 24 hr after axotomy at birth. Scale bar represents 20 μm.

consistent with physiological evidence of low-quantal-content neuromuscular axons in the perinatal period (Colman et al., 1997; Kuno et al., 1971). Subthreshold axonal inputs would be invisible to postsynaptic activity-based assays such as glycogen depletion or muscle tension, explaining the disparity between these results with physiological measures of motor unit size (see Discussion).

No Evidence of Synaptic Specificity

The large number of converging axons raised the possibility that at birth, muscle fibers were innervated by a substantial fraction or perhaps even all of the axons that innervated the region of muscle they resided in. As already described (Figure 3), in some muscles, axons project to a limited region of the endplate band at birth just as they do in later life. From axonal reconstructions at postnatal day 8 from a previous study (Keller-Peck et al., 2001), we analyzed the area of the endplate band occupied by single motor units and found that, on average, axons in the sternomastoid muscle occupied ~18% (0.42 ± 0.12 μm^2, n = 6) of the endplate band area in the muscle as a whole. Because there are in the range of 50–60 primary motor axons innervating the sternomastoid muscle (Nguyen et al., 1998), we anticipate that 18% of these or 9–11 motor axons should project to any one region. This number roughly matches the number of innervating axons per junction at birth, suggesting that, at least in some cases, all the motor axons within the vicinity of a muscle fiber innervate it at birth. Hence, we found no evidence for any synaptic selectivity in the initial innervation pattern as might have been expected if axons preferentially innervated muscle fibers of a particular type.

No Evidence of Neuronal Death or Intermuscular Axon Collateral Loss during Perinatal Synapse Elimination Stage

Although terminal branch loss from large-sized motor units (described above) seemed to be a sufficient explanation for these extra innervating branches, we also tested alternative explanations (Figure 6A). For example, might some of the excess

innervation originate from axons that were sending long collateral branches to multiple muscles in the embryonic period, or, alternatively, might some of the branches originate from motor neurons that are at the tail end of the period of naturally occurring motor neuron cell death and are destined to die? The idea of cell death was ruled out by finding that there were no activated caspase-3 or TUNEL-positive ventral horn cholinergic cells in the spinal cord at birth, even though we could induce caspase-3 or TUNEL labeling in the sternomastoid muscle motor neurons by axotomy in the spinal accessory nerve of pups at P0 (Figure 6C). We also found no evidence of axons branching to more than one muscle at birth by examining both retrograde labeling of motor neurons projecting to different muscles and lipophilic axon tracing from different muscles (Figure 6B).

DISCUSSION

Terminal Branch Pruning

This study shows extensive connectivity in the developing neuromuscular system that resolves over the first few postnatal days into the much simpler pattern that has been well described in previous studies. Motor axons innervate roughly an order of magnitude more target cells, and target cells each receive input from an order of magnitude more axons at birth than 2 weeks later. The loss occurs precipitously because even by postnatal day 6, many of these muscle fibers are singly innervated (Keller-Peck et al., 2001), meaning that the postsynaptic cells must be losing innervation from more than an axon per day during the first postnatal week. This data also shows that the peak of the ''exuberance'' is just before birth, suggesting perhaps that postnatal life may be a critical impetus for this synapse elimination. Although there are many possible reasons for a die off of axonal branches, the studies presented here indicate that neither late apoptosis of a subset of neurons (Landmesser and Pilar, 1974), nor the pruning of long intermuscular axon collaterals that projected erroneously to multiple targets (Bunt and Lund, 1981; Innocenti, 1981; Stanfield et al., 1982), nor the pruning of large intramuscular branches with many synaptic terminals explains the result. Rather, the results show that pruning of terminal synaptic branches explains the large reduction in axonal complexity beginning in the perinatal period.

Anatomy as an Approach to Reveal Weak and Recently Eliminated Synapses

We have studied the excessive branching using light and electron microscopical anatomical methods. Light and electron microscopy were necessary because of technical limitations of electrophysiological and more traditional light microscopic assays when used in developing systems. We measured the size of neonatal motor units anatomically because the several physiological methods previously used are insensitive to subthreshold innervation. One approach measures the muscle tension elicited by individual motor axons and compares it with the total tension a muscle is capable of generating (Brown et al., 1976). A second method stimulates a motor axon in a relatively anaerobic condition to deplete all the glycogen in the activated muscle fibers (Jones and Ridge, 1987; Lichtman and Wilkinson, 1987; Thompson et al., 1984). Both of these physiological measurements argue that shortly after birth, motor units are up to 5-fold larger than they are 2 weeks later but with some already at adult sizes (Bennett and Pettigrew, 1974; Betz et al., 1979; Brown et al., 1976). Because these measurements record the contribution of synapses capable of driving muscle fibers to contract, they will certainly underestimate the actual size of motor units if they contain subthreshold inputs. However, the ''subset''-expressing transgenic mice in which often only a single axon projecting to a muscle is fluorescent when used in association with a postsynaptic label (such as fluorescently tagged alpha bungarotoxin) provides a direct measure of the number of fibers in a motor unit independent of the size of contact.

We also resorted to anatomy to gauge the number of axons innervating a muscle fiber. One standard electrophysiological assay to estimate the number of axons innervating a muscle fiber is to monitor the number of discrete synaptic potentials while gradually increasing the strength of stimulus to the innervating nerve bundle (Redfern, 1970). In muscle, this approach is typically done in the presence of a nonsaturating dose of a cholinergic blocker (e.g., curare) to prevent muscle twitching. As a consequence, the weakest inputs are potentially too small to be detected, leading to an underestimate of the actual number of innervating axons. Moreover, accurate counts of the number of innervating axons by recruitment of synaptic potentials are challenging in young animals because of high quantal variation, low quantal content, and the larger number of axonal inputs (Bennett and Pettigrew, 1974; Chen and Regehr, 2000; Lichtman, 1980). Also confounding physiological measures is the possibility that the synaptic potentials recorded can potentially be due to spillover from nearby synapses on other postsynaptic cells (Takayasu et al., 2006). In addition, physiological methods cannot detect recently eliminated axons. Thus, there was considerable uncertainty concerning the extent of multiple innervation at developing neuromuscular junctions. Because developing axons are small caliber and typically so closely fasciculated that the space between them is below the resolution limit imposed by diffraction, light microscopy was inadequate for a measure of the number of axons converging at neuromuscular junctions. To get a definitive answer to the question of how many axons converge on a young neuromuscular junction, we therefore resorted to serial electron microscopy with 50-fold better lateral resolution (4 nm) and 20-fold better depth resolution (30 nm) than standard light microscopy. The serial electron microscopy reconstructions of neuromuscular junctions and the axonal branching resulting from single fluorescently labeled motor units provide a consistent picture indicating that of the many axons converging at a neuromuscular junction at birth, none are obviously dominant. Instead, most of the connections appear quite weak, occupying only a small percentage of the AChR site. This is a marked contrast from the situation a few days to 2 weeks later, when only one axon occupies all the AChRs at each neuromuscular junction. Thus, the developmental reorganization of axons has two important consequences: many synaptic branches are lost and the remaining synaptic branches become much more powerful. Thus, neurons redistribute their synaptic resources from weakly innervating many target cells to strongly innervating only a few. This reapportionment in developing muscle is analogous to what has been

described with physiological methods in the developing thalamus (Chen and Regehr, 2000) and the parasympathetic nervous system (Lichtman, 1977). However, in both of those situations, the extra synaptic potentials observed in young preparations could at least in part be explained by spillover of neurotransmitter from synapses on adjacent postsynaptic cells. Our anatomical results are not subject to the same uncertainty.

It is important not to discount the significance of the weak inputs. Comparisons of our anatomical data with previous physiological measurements of motor unit size in the mouse (Fladby, 1987) suggest that nearly two-thirds of the innervating axonal branches at birth that we saw would be subthreshold and invisible to functional muscle twitch-based assays. However, these ineffective inputs are crucially related to the outcome of synapse elimination, because at birth, we find that more than 93% of the junctions lack any input that occupies the majority of the junctional area. Thus, from among these weak inputs, one must eventually emerge as the dominant source of innervation. It is likely that this strengthening occurs in large part by an interaxonal competition in which the remaining axon takes over synaptic territory ceded by the axonal branches that are removed (Turney and Lichtman, 2012; Walsh and Lichtman, 2003).

Synapse Elimination: Redistribution of Synaptic Resources

What is the purpose of this large-scale change in connectivity? It is possible that very large motor units assure that all muscle fibers initially receive innervation from all or nearly all the axons that project in their vicinity. Given the wealth of data that suggests that both motor neurons and muscle fibers are molecularly heterogeneous (Jansen and Fladby, 1990), the extensive convergence and divergence may mean that all muscle fibers get access to all motor neuron types, affording maximum flexibility in the establishment of the final pattern of connections. Axons, however, may not have sufficient metabolic capacity to drive to threshold the large number of muscle fibers they initially contact. Thus, the subsequent retrenchment may help guarantee that each axon ends up with an axonal arbor that is small enough to have the capacity to always drive its cohort of postsynaptic targets to threshold—a hallmark of mature neuromuscular junctions. That axonal resources may be in limited supply is supported by the finding that large axonal arbors are more susceptible to axonal branch loss (Thompson and Jansen, 1977) and that sprouting axons in adults incompletely occupy synaptic sites (Schaefer et al., 2005). Moreover, we found that the total volume of axoplasm in a mature motor axon, despite its much smaller number of branches, is greater than the amount of axoplasm within a perinatal axon. This result also suggests that axons may restrict their branch number in compensation for animal growth to maintain functionally effective terminal branches by redistributing resources that are in limited supply. Indeed, what may drive some branches to survive and others to be lost are the relative amount of resources available to each of the innervating axons converging at a neuromuscular junction. When one critical resource, the ChAT enzyme, which synthesizes the neurotransmitter acetylcholine, is experimentally limited in some neurons, they preferentially lose branches when confronting axons with normal levels of ChAT (Buffelli et al., 2003). These results suggest that the large-scale reorganization of motor units described in the present study may ultimately serve to optimize functional connectivity as animals begin to use their muscles.

The evidence we present suggests that local cues at or near synapses determine the outcome of this early phase of axon arbor reorganization. We found that axons in newborn animals can in one case be retracting a branch from one neuromuscular junction while maintaining a branch on an adjacent muscle fiber. This kind of evidence argues that even at the earliest stages of synapse elimination, the signals leading to branch loss are located in the local milieu of the terminal branches. We found no evidence for the alternative idea that neurons were sculpting their nascent axon arbors because of more general shape or positional information considerations. Even the axonal arbors of the functionally homologous motor neuron innervating the same muscle on the left and right side of the same animal have completely different branching patterns (Lu et al., 2009). In contrast, many classes of neurons have dendritic arbors that do mature into stereotyped shapes and occupy stereotyped class-specific territories. The stereotypy of dendrite arbors may indicate that dendrite shape is developmentally regulated in a fundamentally different way than axon shape. One possible reason for the great variability of axonal arbors in muscle is that the potentially large number of permutable interactions among the cohort of ten or so axons co-occupying a neuromuscular junction leads to the sequential pruning of all but one of the axons in early postnatal life, with many potentially different outcomes and therefore different effects on the branching pattern. A deeper understanding of this phenomenon may require separate tagging of each axon (Livet et al., 2007) or serial electron microscopy of whole muscles in order to identify all the axonal connectivities within a young muscle to ultimately glean the rules that determine which synapses survive and which are eliminated during neural circuit development.

Are There Similar Reorganizations Elsewhere in the Nervous System?

The synaptic reorganizations that occur at the neuromuscular junction are exceptional in that the postsynaptic targets, i.e., muscle fibers, are not part of the nervous system per se. Accordingly, are the principles underlying the development of neuromuscular connectivity relevant to the rest of the nervous system? In one sense, muscle fibers are analogous to at least some postsynaptic neurons because in the cerebellum, thalamus, and autonomic ganglia, among other sites, neurons are known to lose axonal inputs at approximately the same developmental stage that motor axons prune (Chen and Regehr, 2000; Lu and Trussell, 2007; Mariani, 1983; Purves and Lichtman, 1980). In another sense, however, there could be significant differences between synaptic reorganization occurring on muscle fibers and neurons because the total number of synapses contacting nerve cells is increasing during development (Huttenlocher, 1979; Zecevic et al., 1989). Whether this is a real difference between neurons and muscle (or just a semantic one—see below) depends on what is the source of the added synapses in the growing brain. For example, if at the time some axons remove all their synapses from a neuron, there are new axonal inputs connecting with target neurons for the first time, then

the net effect might be no change in the number of innervating axons, even if there is an increase in the total number of synapses. To our knowledge, there is no evidence that either strongly supports or refutes the idea of a wave of new axons establishing innervation with a target cell at the postnatal ages when other axons are being eliminated. Alternatively, if at the time some axons remove their connections from a postsynaptic neuron, a subset of axons that already are innervating the same postsynaptic cell establish additional synaptic connections, then the pruning of some inputs could lead to a net reduction in axonal convergence, while the total number of synapses is not affected. In this scenario, the number of synapses is decoupled from the number of axons so that it is even possible that the total synapse number on a target cell actually increases despite the loss of axonal input. In the parasympathetic submandibular ganglion, this is exactly what does happen: as the number of innervating axons per postsynaptic neuron decreases >5-fold, the number of synapses increases ~2-fold, as one of the axons adds synapses to more than compensate for the loss of the other axons (Lichtman, 1977). Similarly, in the developing cerebellum, as the number of climbing fibers innervating a Purkinje cell is reduced, the number of synapses elaborated by the remaining climbing fiber increases (Hashimoto and Kano, 2003; Sugihara, 2005). In the developing thalamus, as the number of retinogeniculate axons innervating individual geniculocortical neurons drops, the synaptic strength of the remaining input rises (Chen and Regehr, 2000), perhaps due to elaboration of new synapses. In all these cases, axon loss could be associated with an increase in synapse number due to additional synaptogenesis from one of the remaining inputs. We think the same theme is also apparent at the neuromuscular junction despite confusion in nomenclature about the word "synapse." Although the adult singly innervated neuromuscular junction is referred to as a "synapse," it is actually a cluster of synaptic release sites. As muscle fibers grow, the postsynaptic area increases and the nerve terminal opposed to it enlarges, probably adding many new release sites (Marques et al., 2000). Recent data suggests that the reason the axons can elaborate new sites is that the presence of recently vacated postsynaptic sites causes nearby synaptic terminals to sprout to innervate the unoccupied acetylcholine receptors (Turney and Lichtman, 2012). Hence, postsynaptic sites are exchanged between axons, with the eliminated axons ceding their sites to the remaining ones and allowing the survivors to increase their quantal content (Colman et al., 1997; Walsh and Lichtman, 2003). Moreover, target muscle fibers and their postsynaptic territories continue to grow so the final result is that the axon that remains has overall many more synaptic release sites than the ten or so axons that converged at birth. If an analogous synaptic exchange occurs in the developing brain, the rise in synapse number observed in the developing central nervous system might mask a loss of axonal inputs that is commensurate with the dramatic events occurring in developing muscle.

EXPERIMENTAL PROCEDURES

An expanded Experimental Procedures section is provided in the Supplemental Experimental Procedures.

Mice

Transgenic mice (Feng et al., 2000) were bred and housed according to the guidelines of the Harvard Animal Care and Use Committee.

Tissue Preparation

Pups were deeply anaesthetized with KX (ketamine/xylazine) and transcardially perfused with 2% paraformaldehyde. Isolated muscles were immunostained using a primary antibody to GFP (Chemicon) and a secondary antibody conjugated with Alexa 488 (Invitrogen).

Imaging

Motor units were imaged with confocal microscopy (Olympus FV-1000) using a 60× PlanAPO (1.4 NA) objective by excitation of Alexa 488 antibody and the Alexa 647-tagged α-bungarotoxin. Care was taken to magnify the images via laser scanning at the diffraction limit to assure that the finest processes were well resolved.

Serial Section Scanning Electron Microscopy and Image Reconstruction

Tissue for electron microscopy was processed as previously described (Hayworth et al., 2006; Tapia et al., 2012). Sections were placed on a silicon wafer and imaged at ~10kV (JEOL 6701F). All montages were aligned and segmented using TrakEM2 in NIH Image (Cardona et al., 2010).

Analysis

Junctional occupancy was determined by the number of colocalized green (axon) and red (AChR) pixels divided by the total number of red (receptors) pixels. Axonal caliber was assessed by measuring the width of each axonal trunk at four random locations. Branch order was determined by constructing a complete branching diagram for the arbor.

Statistics

Statistical comparisons used the unpaired Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2012.04.017.

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REFERENCES

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

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.

Bishop, D.L., Misgeld, T., Walsh, M.K., Gan, W.B., and Lichtman, J.W. (2004). Axon branch removal at developing synapses by axosome shedding. Neuron *44*, 651–661.

Brill, M.S., Lichtman, J.W., Thompson, W., Zuo, Y., and Misgeld, T. (2011). Spatial constraints dictate glial territories at murine neuromuscular junctions. J. Cell Biol. *195*, 293–305.

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

Bruusgaard, J.C., Liestøl, K., Ekmark, M., Kollstad, K., and Gundersen, K. (2003). Number and spatial distribution of nuclei in the muscle fibres of normal mice studied in vivo. J. Physiol. *551*, 467–478.

Buffelli, M., Burgess, R.W., Feng, G., Lobe, C.G., Lichtman, J.W., and Sanes, J.R. (2003). Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature *424*, 430–434.

Bunt, S.M., and Lund, R.D. (1981). Development of a transient retino-retinal pathway in hooded and albino rats. Brain Res. *211*, 399–404.

Cardona, A., Saalfeld, S., Preibisch, S., Schmid, B., Cheng, A., Pulokas, J., Tomancak, P., and Hartenstein, V. (2010). An integrated micro- and macroarchitectural analysis of the Drosophila brain by computer-assisted serial section electron microscopy. PLoS Biol. *8*, e1000502.

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.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D., and Stanfield, B.B. (1984). Regressive events in neurogenesis. Science *225*, 1258–1265.

Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., 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. (1987). Postnatal loss of synaptic terminals in the normal mouse soleus muscle. Acta Physiol. Scand. *129*, 229–238.

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

Hashimoto, K., and Kano, M. (2003). Functional differentiation of multiple climbing fiber inputs during synapse elimination in the developing cerebellum. Neuron *38*, 785–796.

Hayworth, K.J., Kasthuri, N., Schalek, R., and Lichtman, J.W. (2006). Automating the collection of ultrathin serial sections for large volume TEM reconstructions. Microsc. Microanal. *12*, 86–87.

Huttenlocher, P.R. (1979). Synaptic density in human frontal cortex - developmental changes and effects of aging. Brain Res. *163*, 195–205.

Innocenti, G.M. (1981). Growth and reshaping of axons in the establishment of visual callosal connections. Science *212*, 824–827.

Jacobson, M. (1969). Development of specific neuronal connections. Science *163*, 543–547.

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

Jones, S.P., and Ridge, R.M. (1987). Motor units in a skeletal muscle of neonatal rat: mechanical properties and weak neuromuscular transmission. J. Physiol. *386*, 355–375.

Keller-Peck, C.R., Walsh, M.K., Gan, W.B., Feng, G., Sanes, J.R., and Lichtman, J.W. (2001). Asynchronous synapse elimination in neonatal motor units: studies using GFP transgenic mice. Neuron *31*, 381–394.

Kuno, M., Turkanis, S.A., and Weakly, J.N. (1971). Correlation between nerve terminal size and transmitter release at the neuromuscular junction of the frog. J. Physiol. *213*, 545–556.

Landmesser, L., and Pilar, G. (1974). Synaptic transmission and cell death during normal ganglionic development. J. Physiol. *241*, 737–749.

Lichtman, J.W. (1977). The reorganization of synaptic connexions in the rat submandibular ganglion during post-natal development. J. Physiol. *273*, 155–177.

Lichtman, J.W. (1980). On the predominantly single innervation of submandibular ganglion cells in the rat. J. Physiol. *302*, 121–130.

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

Lichtman, J.W., and Wilkinson, R.S. (1987). Properties of motor units in the transversus abdominis muscle of the garter snake. J. Physiol. *393*, 355–374.

Livet, J., Weissman, T.A., Kang, H., Draft, R.W., Lu, J., Bennis, R.A., Sanes, J.R., and Lichtman, J.W. (2007). Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature *450*, 56–62.

Lu, T., and Trussell, L.O. (2007). Development and elimination of endbulb synapses in the chick cochlear nucleus. J. Neurosci. *27*, 808–817.

Lu, J., Tapia, J.C., White, O.L., and Lichtman, J.W. (2009). The interscutularis muscle connectome. PLoS Biol. *7*, e32.

Lupa, M.T., and Hall, Z.W. (1989). Progressive restriction of synaptic vesicle protein to the nerve terminal during development of the neuromuscular junction. J. Neurosci. *9*, 3937–3945.

Mariani, J. (1983). Elimination of synapses during the development of the central nervous system. Prog. Brain Res. *58*, 383–392.

Marques, M.J., Conchello, J.A., and Lichtman, J.W. (2000). From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. J. Neurosci. *20*, 3663–3675.

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.

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

Redfern, P.A. (1970). Neuromuscular transmission in new-born rats. J. Physiol. *209*, 701–709.

Riley, D.A. (1981). Ultrastructural evidence for axon retraction during the spontaneous elimination of polyneuronal innervation of the rat soleus muscle. J. Neurocytol. *10*, 425–440.

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

Schaefer, A.M., Sanes, J.R., and Lichtman, J.W. (2005). A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. J. Comp. Neurol. *490*, 209–219.

Stanfield, B.B., O'Leary, D.D., and Fricks, C. (1982). Selective collateral elimination in early postnatal development restricts cortical distribution of rat pyramidal tract neurones. Nature *298*, 371–373.

Sugihara, I. (2005). Microzonal projection and climbing fiber remodeling in single olivocerebellar axons of newborn rats at postnatal days 4-7. J. Comp. Neurol. *487*, 93–106.

Takayasu, Y., Iino, M., Shimamoto, K., Tanaka, K., and Ozawa, S. (2006). Glial glutamate transporters maintain one-to-one relationship at the climbing fiber-Purkinje cell synapse by preventing glutamate spillover. J. Neurosci. *26*, 6563–6572.

Tapia, J.C., and Lichtman, J.W. (2013). Synapse elimination. In Fundamental Neuroscience, Fourth Edition, L. Squire, D. Berg, F.E. Bloom, S. du Lac, A. Ghosh, and N.C. Spitzer, eds. (Amsterdam: Academic Press), in press.

Tapia, J.C., Kasthuri, N., Hayworth, K.J., Schalek, R., Lichtman, J.W., Smith, S.J., and Buchanan, J. (2012). High-contrast en bloc staining of neuronal tissue for field emission scanning electron microscopy. Nat. Protoc. *7*, 193–206.

Thompson, W., and Jansen, J.K. (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.

Turney, S.G., and Lichtman, J.W. (2012). Evidence for synaptic competition and its mechanism. PLoS Biol., in press.

Walsh, M.K., and Lichtman, J.W. (2003). In vivo time-lapse imaging of synaptic takeover associated with naturally occurring synapse elimination. Neuron *37*, 67–73.

Zecevic, N., Bourgeois, J.P., and Rakic, P. (1989). Changes in synaptic density in motor cortex of rhesus monkey during fetal and postnatal life. Brain Res. Dev. Brain Res. *50*, 11–32.