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Title: **PREVALENCE AND ELIMINATION OF SIBLING NEURITE CONVERGENCE IN MOTOR UNITS SUPPLYING NEONATAL AND ADULT MOUSE SKELETAL MUSCLE.**

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Abbreviated Title: **Sibling neurite convergence in motor units**.

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ABSTRACT

During development neurons form supernumerary synapses, most of which are selectively pruned leading to stereotyped patterns of innervation. During the development of skeletal muscle innervation, or its regeneration after nerve injury, each muscle fiber is transiently innervated by multiple motor axon branches but eventually by a single branch. The selective elimination of all but one branch is the result of competition between the converging arbors. It is thought that motor neurons initially innervate muscle fibers randomly, but that axon branches from the same neuron (sibling branches) do not converge to innervate the same muscle fiber. However, random innervation would result in many neonatal endplates that are co-innervated by sibling branches. To investigate whether this occurs we examined neonatal levator auris longus (LAL) and 4th deep lumbrical (4DL) muscles, as well as adult reinnervated deep lumbrical muscles (1-4) in transgenic mice expressing yellow fluorescent protein (YFP) as a reporter. We provide direct evidence of convergence of sibling neurites within single fluorescent motor units, both during development and during regeneration after nerve crush. The incidence of sibling neurite convergence was 40% lower in regeneration and at least 75% lower during development than expected by chance. Therefore, there must be a mechanism that decreases the probability of its occurrence. As sibling neurite convergence is not seen in normal adults, or at later time-points in regeneration, synapse elimination must also remove convergent synaptic inputs derived from the same motor neuron. Mechanistic theories of synaptic competition should now accommodate this form of isoaxonal plasticity.

INTRODUCTION

In the developing nervous system, neurons initially form excessive connections, which are subsequently eliminated through withdrawal of synapses and pruning of axon collaterals. These processes refine central and peripheral connections. Specifically, for motor neurons it is assumed that motor axons are guided by molecular cues and geometric constraints to innervate the correct muscle (Jacob et al., 2001; Jansen and Fladby, 1990; Jacobson, 1978) but within that muscle, muscle fibers are initially innervated randomly (Willshaw, 1981; Barber and Lichtman, 1999;

Rasmussen and Willshaw, 1993). This results in innervation of every muscle fiber by several axon branches. Synapse elimination proceeds by competition between converging inputs until all but one have been eliminated, concluding with a single motor axon branch innervating each muscle fiber. The outcome of this competition is evidently influenced by motor neuron activity (Ribchester and Taxt, 1983; Ridge and Betz, 1984; Callaway et al., 1987; Barry and Ribchester, 1995; Costanzo et al., 2000; Personius et al., 2007) and a hierarchy within motor neurons (Kasthuri and Lichtman, 2003) possibly based on differences in synaptic strength (Colman et al., 1997; Buffelli et al., 2003). Competition at the neuromuscular junction (NMJ) has always been studied under the assumption that competing arbors belong to different neurons and are distinguishable from each other. We investigated here a finding that two or more axon branches deriving from the same motor neuron can innervate the same muscle fiber. We have termed this phenomenon 'sibling neurite convergence' (SNC), alluding to 'sibling neurite bias' as discussed by Smalheiser and Crane (1984). SNC has not been reported in the literature in studies where intra-muscular arbors were traced in adulthood (Lu et al, 2009, Murray et al., 2010) or late development (Keller-Peck et al., 2001; Kasthuri and Lichtman, 2003). However, we calculated that random innervation would lead to frequent instances of it. This, therefore, raises some questions: Do sibling neurites converge on the same endplate or are there constraints that prevent this from happening? If they do, can they eliminate each other or are they maintained? Formal models of developmental synapse elimination assume that sibling neurites do not innervate the same endplate and do not discuss whether they could accommodate competitive within-unit synapse elimination.

We present compelling examples of SNC, both during neuromuscular development and during reinnervation of denervated adult muscle, an accessible and commonly exploited paradigm for modelling synapse elimination in development (Brown et al., 1976; Rich and Lichtman, 1989; Costanzo et al., 2000). The incidence in both types of synaptic maturation is lower than expected by chance. Nonetheless, those endplates initially receiving sibling branches ultimately become innervated by only one axon branch. The data therefore suggest that within-unit convergence does occur and that sibling axon branches are selectively eliminated but, since the incidence of SNC was lower than expected, there must also be constraints on synapse formation between axon branches belonging to the same neuron.

These observations and analysis therefore place important constraints on plausible mechanisms of synapse elimination. Discovering mechanisms that inhibit or eliminate SNC would therefore provide insight into the process of innervation of neural structures.

METHODS

All animal procedures were carried out in accordance with UK Home Office regulations. Thy1-YFP16/C57BL6 (YFP16) and thy1-YFPH/C57BL6 (YFPH) mice were originally obtained from Jackson Labs (Bar Harbor, Maine) and used to establish in house breeding colonies.

Table 1. Primary antibody

Antibody	Immunogen	Source/cat number	species	Dilution for IHC	Reference
Anti-GFP	Highly purified native GFP from Aequorea victoria	Millipore/AB3080	Rabbit polyclonal	1:1000	Schaefer et al. (2005)

Neonatal LAL motor units

Neonatal (p5-p6) YFPH pups were pups were anaesthetised in a closed chamber whose atmosphere was equilibrated with halothane from a liquid halothane-saturated towel and then sacrificed by overdose of anaesthetic and decapitated. The LAL muscle was dissected by making a small incision between the eyes and carefully cutting the skin down the mid-line of the head and around the ears. Connective tissue was cut away until the LAL muscle attached still to the ears could be removed and then pinned into a Sylgard coated dish containing mammalian physiological solution (MPS; 120 mM NaCl; 5 mM KCl; 2 mM CaCl2; 1 mM MgCl2; 0.4 mM NaH2PO4; 23.8 mM NaHCO3 and 5.6 mM D-glucose, equilibrated by bubbling with 95% O2/5% CO2; pH 7.2-7.4) before fixing and staining. AChR were stained with 5µg/ml TRITC-α-BTX (Invitrogen or Biotium, Inc.) in MPS and fixed in 4% paraformaldehyde (PFA; Fisher Scientific) in 1% phosphate buffered saline (PBS; pH 7.2-7.4). The YFP signal was amplified by immunostaining. For this, muscles were incubated with permeabilising solution consisting of 4% bovine serum albumin (Sigma) and 0.5% Triton-X

(Sigma) in PBS for 30 minutes at room temperature. Then, samples were incubated with 1:1000 by volume of the primary antibody (anti-GFP, Millipore, Table 1) in blocking solution overnight at room temperature. This primary antibody has been shown to selectively bind to fluorescent protein, as animals that do not transgenically express fluorescent protein do not show staining (Schaefer et al., 2005). After incubation with the primary antibody samples were given 2x10 minute washes in PBS and then incubated in 1:1000 by volume secondary antibody (swine-anti-rabbit-FITC, Dako or anti-rabbit-488, Jackson Labs) in PBS for 2-4 hours at room temperature. Finally, samples were washed in PBS at least 3 times for at least 10 minutes. After further dissection muscles were mounted on glass slides for confocal microscopy. Muscles with a single fluorescent unit (nonfluorescent units were also present in these preparations) were imaged on a Zeiss 510 inverted confocal microscope using a 63x 1.4 NA oil objective.

Neonatal lumbrical motor units

Neonatal (p5) YFP16 pups were anaesthetised by chilling and the tibial nerve was cut bilaterally, causing partial denervation of the 4th deep lumbrical (4DL) muscle (Betz et al., 1980). Three days later (p8) pups were sacrificed by cervical dislocation and the 4DLs were dissected, stained with TRITC- α -BTX and fixed in PFA. YFP signal was amplified with anti-GFP immunolabelling and muscles were mounted on glass slides as described above. Muscles with a single remaining motor unit (no non-fluorescent units were present in these preparations) were selected for further imaging with a Zeiss LSM510 inverted confocal microscope using a 63x 1.4 NA oil objective.

Regenerating lumbrical motor axons

Adult (8-46 weeks, mean age: 21 weeks) YFPH mice were anaesthetised with Halothane (2-5% in 1:1 O2/*N*2*O*; Merial Animal Health Ltd) or Isofluorane (2-5% in O2) and the lumbrical muscles of both hind feet were completely denervated by crushing the tibial and sural nerves. Mice were allowed to recover either 12-35 days or more than 70 days and compared with control unoperated mice (6-17 weeks, mean age: 12 weeks). Mice were sacrificed by cervical dislocation and the deep lumbrical muscles 1-4 were removed, stained with TRITC- α -BTX, fixed in PFA and mounted on glass slides as described above. Muscles with a single fluorescent motor unit (non-fluorescent units were also present in these preparations) were selected for further analysis. These muscles were

imaged using a Biorad Radiance 2000 confocal microscope and almost every innervated endplate was captured using a 40x 1.3 NA oil lens.

Image Analysis

All images were analysed in ImageJ (Rasband, 2009, available from:http://rsbweb.nih.gov/ij/), Fiji ('Fiji Is Just ImageJ' pre-packaged with image processing software, http://pacific.mpi-cbg.de), Adobe Photoshop and Gimp (GNU Image Manipulation Program, http://www.gimp.org/). Axons were traced and axon length measured using the 'Simple Neurite Tracer' plugin for Fiji. Branch lengths were not normally distributed (Kolmogorov-Smirnov test) and so the reciprocal was used for statistical testing.

Diameter was estimated by measuring the width of the axon, using the ImageJ line tool. Montages were made using the 'Stitching' plugin in Fiji and by hand in Gimp and Adobe Photoshop. In all images color range levels were uniformly changed for display purposes but no other manipulations were made.

Statistical Analysis

Statistical tests were performed in R or SPSS. Quoted values are mean ± SD unless otherwise stated.

Mathematical Analysis

In the following analysis we derive an equation which takes two inputs: (1) the total number of muscle fibers in a muscle, and (2) the motor unit size of a given motor unit, and calculates the number of muscle fibers that will be convergently innervated by two or more branches of the particular motor unit. Here we assume that all branches of the motor unit are equally likely to innervate any muscle fiber (and, conversely, all muscle fibers are equally likely to be innervated) and that the probability of any branch innervating a muscle fiber does not depend on any of the other branches.

Suppose a motor axon has B branches, each of which randomly and independently innervates one of

M muscle fibers. The number of muscle fibers with two or more converging inputs from this same motor neuron will equal the total number of muscle fibers minus the number of muscle fibers that are innervated by either none or exactly one of the *B* branches. If each muscle fiber has an equal probability of being innervated, the probability that any given branch bi will innervate a given muscle fiber m_j is $\frac{1}{M}$. Therefore the probability that m_j is not innervated by branch b_i b_i is $1-\frac{1}{M}$. Since there are *B* branches, the probability of a muscle fiber not being innervated by any branch is $(1-\frac{1}{M})^B$. Thus, the number of muscle fibers that will be innervated by no branches is given by

$$M_0 = M \times (1-\frac{1}{M})^B$$

The probability of a given muscle fiber m_j being innervated by the first branch (b_1) and none of the others is equal to $\frac{1}{M} \times (1-\frac{1}{M})^{(B-1)}$. There are B different ways in which an endplate can become innervated by a single branch (one for every branch), so the total probability of an endplate being contacted by exactly one branch is $B \times \frac{1}{M} \times (1-\frac{1}{M})^{(B-1)}$. The number of endplates which will be innervated exactly once will be

$$M_1 = M \times B \times \frac{1}{M} \times (1-\frac{1}{M})^{(B-1)} = B \times (1-\frac{1}{M})^{(B-1)}$$

Therefore the expected number of muscle fibers innervated by converging sibling branches is

$$M_2 = M - M \times (1-\frac{1}{M})^B - B \times (1-\frac{1}{M})^{(B-1)} \qquad \text{Equation 1}$$

Simulation

We performed a simulation in MATLAB to show the consequence of muscle fibers within a muscle having different probabilities of being innervated. We assumed 250 muscle fibers and a motor unit of size 150. These values match what we found in neonatal mouse lumbrical muscles. For each round of the simulation we first assigned a probability to each muscle fiber that reflected the likelihood that it would be innervated by any branch. Then for each of the 150 axon branches we used these probabilities to randomly selected a muscle fiber for to be innervated. Once all 150 axon branches had been assigned a muscle fiber, we counted the number of fibers contacted by two or more of the axon branches. For each probability assignment we repeated this process 100 times and

plotted the average number of muscle fibers contacted by two or more branches. We used 40 different probability assignments that varied between all muscle fibers having equal probability of being innervated (uniform) to some muscle fibers having a 40x greater chance of being innervated than others (skewed).

RESULTS

If the initial innervation pattern were random, sibling neurite convergence would occur frequently. We derived Equation 1, which calculates exactly how often this should occur within a motor unit (MU) with B branches, which innervates a muscle with M innervation sites (that is, muscle fibers). We can directly measure the MU size and the number of muscle fibers in a particular specimen and therefore calculate the amount of sibling neurite convergence (SNC) expected for that particular unit. Thus, this equation does not make assumptions about the distribution of motor unit sizes within a muscle. It does assume that each muscle fiber has an equal probability of being innervated.
In reality, muscle fibers may have different probabilities of being innervated. We have shown by simulation that the expected amount of SNC increases as the variability in the innervation probability of muscle fibers increases (Figure 1E). There are two conclusions of interest to note. First, by assuming equal innervation probabilities of all muscle fibers we have used a conservative estimate of the amount of SNC expected. Second, the difference between a 40-fold variance in innervation probabilities and an equal innervation probability is only 4 instances of SNC (30 to 34 for the given MU size and number of muscle fibers used here), therefore our estimate is likely to be close to the true value, even under the simplifying assumption that we have made.

In general, the more branches a motor axon has and the fewer muscle fibers, the higher the expected incidence of sibling neurite convergence. Figure 1 shows the amount of SNC expected for different MU sizes in four different muscles. The total number of muscle fibers and the average neonatal motor unit sizes for each muscle are based on published data and our unpublished observations (Thompson and Jansen, 1977; Betz et al. 1979; Fladby and Jansen, 1987). According to this analysis all four of these muscles should have on the order of 8-75 instances of SNC in each motor unit of

average size.

In order to estimate the amount of SNC expected in a whole muscle we multiplied the estimated number of SNC in each average unit by the number of motor units in a given muscle. This estimate is not exactly accurate because MU sizes can vary within a muscle. However, adding in variance in MU sizes, while keeping the average MU size constant, increases the amount of SNC expected as can be seen in Figure 1D. This is because the amount of SNC added by having some larger units is more than the amount lost by having some smaller units.

Sibling branches infrequently converged on the same endplate during development

There were four clear instances of convergently innervated endplates in the LAL muscle (Figure 2A-D). In addition, there were indications of the same phenomenon in developing lumbrical muscle (Figure 2E&F).

We examined five single motor units in the LAL muscle from three different YFPH line mice aged p5-p6. In this line fluorescent protein is expressed in a subset of motor neurons. The axonal trees that were traced all derived from a single branch and therefore belonged to the same unit, although it is possible that the motor unit had branches in other regions that were not traced. Each motor unit innervated between 15 and 61 endplates (see Table 2). Four of the 129 innervated endplates were convergently innervated by sibling branches and three of these were part of the same motor unit (Figure 2). The fourth was part of a different unit and one of the converging branches appeared to be a terminal branch from a different endplate. Additionally, there was a fifth endplate that was partially occupied by two very short branches of the fluorescent axon (Figure 2, endplate 14). This suggests that a different (non-fluorescent) axon collateral was also innervating this endplate. All other endplates were partially or fully occupied by a single fluorescent branch.

The fluorescent protein in YFPH line mice is detectable relatively late postnatally in the lumbrical muscles. We did not observe any fluorescence in the lumbrical muscles of YPFH line mice up to p10, even after attempting to amplify the fluorescent signal with anti-GFP antibodies. Thus, in order

to observe single lumbrical neonatal units, 4th deep lumbrical (4DL) muscles from YFP16 mice - in which all motor neurons are fluorescent even at birth - were partially denervated, by cutting the tibial nerve/LPN at p5. This left some muscles innervated by single fluorescent axons supplied by the sural nerve (Betz et al., 1980). The partially denervated muscles were imaged three days later, at p8. The three day delay was sufficient for the arbors of the damaged tibial nerve axons to fully degenerate, allowing us to observe the full arboreal extent of the intact sural nerve units. This was the earliest time-point in development at which we could resolve most (but not all) axon branches, even though there was only a single unit in these muscles. We imaged two 4DL muscles with a single remaining unit at high resolution. As expected at this post-natal stage, both motor units innervated more than 70% of the muscle (see table 2). Most endplates appeared to be innervated by a single branch. Inspection of each endplate provided no compelling examples of sibling convergence. However, there were up to 20 endplates (out of the 375) for which the innervation pattern was not sufficiently resolvable, four of which provide equivocal examples of sibling neurite convergence (see Figure 2F).

The incidence of sibling neurite convergence during development was lower than predicted by chance.

During development there were fewer examples of sibling neurite convergence than we predicted from Equation 1. Table 2 shows both the expected and observed number of muscle fibers innervated by converging sibling branches for each neonatal muscle. Motor unit size equates to the number of muscle fibers that a motor neuron innervates, but is not necessarily equal to the number of branches, since some branches may innervate the same muscle fiber.

For the LAL, only endplates in the innervated region of the muscle were considered as potential innervation sites because this is a segmental muscle and each axonal tree is restricted to one portion of the muscle (Murray et al., 2008). The expected amount of convergent innervation varied between 1 and 9 endplates. In total, the expected amount of convergent innervation (19 endplates) is almost five times greater than that observed (4 endplates); however, this difference was not statistically

significant (Wilcoxon signed rank sum test, ns).

On the other hand, the 4DL is not a segmental muscle and therefore every muscle fiber was considered a possible innervation site. Using Equation 1, we expected approximately 100 endplates in each muscle to be convergently innervated. In fact, in the two muscles we examined there were hardly any indications of this phenomenon. There were none in the first and four equivocal examples in the second, as well as 16 endplates with unresolvable innervation. Even if all 20 ambiguous endplates were examples of sibling neurite convergence, that is still much less than expected. It seems unlikely that the lack of converging branches was entirely due to selective elimination prior to p8, given that polyneuronal innervation in the mouse lumbrical persists until at least p11 and most likely beyond.

These data make clear two important points: (i) sibling neurite convergence does occur during development, at least in the LAL muscle; (ii) the frequency with which it occurs is less than expected assuming random innervation.

One striking feature of the neonatal innervation pattern in both muscles was that, while axon branches belonging to different neurons tended to fasciculate, when there was a single axon, branches did not travel along the same paths (see Figure 3). A mechanism that prevented fasciculation in sibling axon branches could cause them to innervate different regions of the muscle and, thus, reduce the incidence of SNC.

Sibling branches did not converge in unoperated adults

Sibling neurite convergence is not present in adult muscles. We examined 300 NMJs from six different unoperated YFPH adult lumbrical muscles (Figure 1B&C). There were no instances of sibling neurite convergence. This means that the instances we observed in the neonate must have been eliminated during the developmental process.

In order to investigate sibling neurite convergence more systematically and from earlier in the synaptic formation process we turned to regeneration. This is an interesting phenomenon in itself but reinnervation has frequently provided insights into mechanisms of development (Brown et al., 1976; Brown and Ironton, 1978; Ribchester and Taxt, 1983; Rich and Lichtman, 1989; Costanzo et al., 2000).

Adult muscles have some instances of short pre-terminal branches (Figure 1C), which have been described previously (Tuffery, 1971; Harris and Ribchester, 1979) and are thought to be the result of local remodelling at the adult NMJ, either presynaptically or following remodelling of the distribution of acetylcholine receptors (Balice-Gordon & Lichtman 1993). However, what we focused on here was not this close-quarter division of motor nerve terminals but rather, convergent innervation arising from distance that corresponded to several nodes of Ranvier more proximal to the last heminode. Here we are interested in sibling converging branches and not pre-terminal branches. We distinguish between the two by length and by morphology. We measured the length of the axon from the common branch point to the endplate for all branches that converge on the same muscle fiber (without labelling them as pre-terminal or SNC; see figure 1C). We expected preterminal branches to be short but converging sibling branches to consist of a mixed population of short and long branches. In the control animals we found 45 examples (15%) of pre-terminal branches with a mean length of 8.6 ± 6.6 μm from the pre-terminal branch point to the synapse (Range: 2-30 μm, Figure 1C) and 255 (85%) NMJs that had a single branch innervating the endplate. An additional 67 endplates were excluded from further analysis due to insufficient resolution.

Adult sibling branches converged on the same endplate during early but not late stages of regeneration.

When axons are damaged very close to their muscle entry point, reinnervation of endplates occurs following rapid and accurate axon regeneration through pre-existing endoneurial tubes and there is no polyneuronal innervation (Nguyen et al., 2002). However, crush injury centimetres from the

nerve entry point evidently renders muscle fibres receptive to polyneuronal innervation, as in development (Boeke, 1916; 1932). Thus, following axon regeneration after a more proximal nerve crush, motor axons form excess synapses, as in development, leading to polyneuronal innervation, which is then eliminated again resulting in mononeuronal innervation (McArdle, 1975; Brown and Ironton, 1978; Ribchester and Taxt, 1983; Ribchester, 1988b; Rich and Lichtman, 1989). Numerous studies have taken advantage of this apparent recapitulation of the postnatal developmental phenomenon of convergence and its remodelling to seek insight into mechanisms of synapse elimination (Brown & Ironton, 1978; Ribchester & Taxt, 1983; Rich & Lichtman, 1989; Barry & Ribchester, 1995; Costanzo et al 1999,2000). We therefore investigated whether sibling neurite convergence occurs during the phase of polyneuronal innervation in reinnervated adult lumbrical muscles. The tibial and sural nerves of adult YFPH line mice were crushed near the ankle, causing Wallerian degeneration of the distal axons. Nerves were allowed to regenerate between 12-131 days, before sacrificing the mice and observing the first to fourth lumbrical muscles. Muscles with a single fluorescent axon were selected for further analysis and so any converging branches observed would certainly have originated from the same axon. Non-fluorescent (and therefore non-visible) regenerating axons were also present in these muscles.

The operated mice were split into two groups, reflecting different stages of nerve regeneration. Recovery times were 12-35 days (early regeneration, dynamic morphology, polyneuronal innervation still present) and more than 70 days (late regeneration, static morphology, mostly mononeuronally innervated endplates). This distinction was corroborated by examining the number of partially occupied endplates, defined as those with less than 90% occupancy by the fluorescent axon in each group. Approximately 20% (11/58) of endplates were partially occupied in early regeneration, compared with no partially occupied endplates in the control group and 3% (1/32) in late regeneration. (These numbers are based only on endplates with pre-terminal or converging sibling branches.)

We compared the lengths and morphologies of converging branches in the control group with those in early and late regeneration (Figure 4 and Table 3). In early regeneration there was a much wider

range of branch lengths, and the average length (27.5 ± 35.2 Qm) was significantly longer than both control (8.6 ± 6.6 μ m, p<0.001 Dunnett's T3 after ANOVA) and late regeneration lengths (12.2 ± 9.3 μ m, p=0.035, Dunnett's T3 after ANOVA). The branch lengths in control and late regeneration were not significantly different from each other. The histograms in Figure 4A make clear that there are long (over 50 μ m) converging branches in early regeneration that are absent in controls and at later time points. In addition to being longer, some of the branches in early regeneration were morphologically different to those seen in the other groups. These included 15 instances where one of the converging collaterals was a sub-branch of the axonal arbor at the common branch point, i.e. the axon branched one or more times between the common branch point and the convergently innervated endplate (see figure 4D), and the other sub-branch innervated a different endplate. This type of morphology was never seen in the control group and only twice in late regeneration. There were also frequent instances where an endplate was innervated by both a nodal and a terminal branch belonging to the same axon. Most of these were not included in the length measurements because it was not possible to determine which endplate the terminal branch had originated from and which one it was innervating. This type of morphology was also never observed in the control group and only once in late regeneration. Figure 5 shows the branching pattern of an entire motor unit in the early stage of regeneration. This motor unit exhibits both of the unusual morphological features described above. For instance, endplate 28 is innervated by one axon branch that has branched three times since the common branch point and a second that is a terminal branch from endplate 23.

Based on these data we conclude that sibling neurite convergence occurs during the early stage of regeneration but that sibling branches are competitively eliminated, as they are not seen at later time points. In contrast short pre-terminal branches exist throughout reinnervation and in control animals. It is possible that some sibling input elimination is not due to competitive elimination of one branch by its sibling, but rather elimination of both siblings by a different neuron (which would not be visible in these experiments). However, the data do not support this interpretation, since some developing or regenerating end plates were completely innervated by the fluorescent neuron, with convergent sibling branches. For instance, in Figure 4B the end plate was clearly and fully

occupied by the two branches of the same axon (see Discussion, page 17).

There was no correlation between the length of converging branches and the ratio of their diameters in adult control and regenerating motor neurons

Axon thinning has been shown to precede synapse elimination (Keller-Peck et al., 2001; Walsh and Lichtman, 2003) and some of the long converging branches in early regeneration seemed to be innervated by a thin and a thick axon branch. We therefore examined whether there was a difference in the ratio of the diameters (largest/smallest) of converging sibling branches, a possible indication that one branch was in the process of being eliminated. First we tested whether there was a correlation between branch length and diameter ratios, since pre-terminal branches were short and might have more equal diameters than long converging branches, where one branch was destined to be eliminated. However, there was no significant correlation between the average branch length and the ratio of the diameters of each branch (Spearman's rho test, ns, see figure 4E). Next we tested whether there was a difference in the ratio of diameters between the different groups, as elimination was only expected to be occurring in the early regeneration group, so it might have higher ratios than the control and late regeneration group. Again there was no difference in the median ratios between the three groups ($P>0.05$; Kruskal-Wallis test).

The frequency of convergence during early regeneration was less than expected by chance

In early regeneration there were significantly fewer endplates with converging sibling branches than predicted by Equation 1 ($p= 0.038$, Wilcoxon signed rank test). This is despite the fact that our estimate from equation 1 is conservative, as discussed above and we have inevitably overestimated the observed amount of SNC by not excluding the pre-terminal branches. It is unlikely that the low incidence of convergence is entirely due to selective elimination because the incidence did not decrease between 14 days and 35 days. The data therefore suggest that in regeneration, as in development, there is a mechanism that decreases the probability of sibling neurite convergence but does not prevent it.

DISCUSSION

We have shown that SNC occurs in development but not in adults and also that it occurs in early regeneration but not at later stages. Therefore, we conclude that sibling branches selectively eliminate each other. However, the incidence of convergence in both types of synaptic maturation is lower than predicted by chance. Therefore, we also conclude that there is a mechanism that reduces but does not prevent the occurrence of SNC. The existence of SNC, although rare, is important because it challenges our assumptions of the mechanisms of synaptic competition. We have described and measured for the first time, to the best of our knowledge, the phenomenon of SNC and additionally we have placed some quantitative boundary conditions on its prevalence in development and reinnervation. This lays the foundation for future studies to elucidate the mechanisms of how sibling neurites constrain and compete with each other. The present data also indicate that competition between convergent sibling branches must be taken into account when investigating mechanisms or creating quantitative models of synapse elimination.

Axons do not innervate randomly

The prevalence of SNC was less than predicted by chance in both development and in early regeneration. In particular, in developing 4DL muscle more than 70% of muscle fibers were innervated by a fluorescent motor unit with very few indications of convergently innervated endplates. That extent of innervation without sibling neurite convergence strongly suggests that there is a mechanism that impedes this from happening. Likewise, in early regeneration the incidence was significantly lower than predicted by chance even though some of the endplates with converging branches were due to pre-terminal branches. The prevalence was also lower in developing LAL muscle, with three out of the five units examined not having any convergently innervated endplates, although the difference was not significant.

In our calculation we assumed that all muscle fibers are equally likely to be innervated by each axon, although this is not necessarily the case. For example, muscle fibers that develop earlier could have a higher probability of becoming innervated by any individual axon. The model can be extended by assigning different probabilities of contact to different muscle fibers, or to different

motor neurons, to reflect, for example, the fact that adult motor unit sizes can vary significantly (Betz et al, 1979). However, we have shown that in both cases this would increase the predicted level of sibling neurite convergence and thereby widen even more the discrepancy between model prediction and experimental result.

The prediction does not take into account any elimination that has occurred prior to when we observed the muscle. We imaged the earliest resolvable time-points during development and although we believe that some sibling neurite elimination will have occurred at very early stages, before we can measure it, we do not think that this can account for the total difference between the observed and predicted incidences of SNC. In the developing lumbrical muscle, for example, we predicted there should be about 100 instances in each muscle and there was none in one muscle and up to a maximum of 20 in the other. Despite this, the motor unit sizes were large and elimination was ongoing at the time of partial denervation. Assuming elimination proceeds at a constant rate from birth until p11, which is the oldest stage at which we have observed polyneuronal innervation in the mouse lumbrical muscle, we would expect 8/11, or 73% of elimination to have occurred by p8. That leaves 27% of the synapses still present to be eliminated: that is, for neonatal lumbrical muscle, even if 73% of the elimination had already occurred there should still be about 27 endplates in each muscle that were convergently innervated. This was clearly not the case even if all the ambiguous cases were indeed instances of sibling neurite convergence. All 100 converging axons could have been eliminated by p8 only if sibling neurite competition proceeded at a faster rate than between-unit competition. There is no evidence that this would be the case. In fact, we might expect sibling neurites to be eliminated slower than average since they would presumably have the same activity patterns, and differences in activity are known to influence elimination. However, these could also be eliminated by some activity-independent mechanism, as demonstrated using the wellestablished model of reinnervation as a paradigm (Barry & Ribchester, 1995; Costanzo et al., 2000).

In addition, in the adult regenerating lumbrical muscles the incidence of SNC after one month was no lower than after two weeks. Specifically there were about double the number of long branches at

one month, which supports the conclusion that the lower incidence cannot be accounted for only by selective elimination.

This strongly suggests that sibling neurites do not innervate independently of each other. The mechanism which creates the dependency between the branches could be something as simple as a rule for how often a neurite will branch, or it could involve some form of self-recognition. Our observation that sibling neurites do not appear to fasciculate during development is interesting in this context. If branches of the same neuron do not fasciculate, causing them to terminate in different parts of the muscle, this may lead to sibling axons having different probabilities for innervating muscle fibers and thus not converging as often as expected under the random model. This observation is corroborated by examining the motor units in Lu et. al. (2009) in which individual branches of the same axons were not generally fasciculated. What causes the lack of fasciculation is unclear. One possible mechanism could be recognition or repulsion between sibling branches, as has been shown to be the case for other types of neurons. For example leech comb cells grow into intricate comb-like patterns without any overlap between processes from the same neuron (Baker and Macagno, 2007). This is achieved through contact-mediated retraction. Likewise, hippocampal dendrites have been shown to grow away from their own soma, while being unaffected by the somata of neighbouring cells (Samsonovich and Ascoli, 2003). The shape of neurons influences the way each integrates and transmits information; therefore the mechanisms and constraints by which a neuron grows could have important implications for the processing it is capable of.

Sibling axons are selectively eliminated.

Although the incidence of SNC was lower than expected by chance, it does occur and the present data provide strong evidence that isoaxonal, sibling branches are selectively eliminated. The converging sibling branches we found in development and early regeneration were not just longer but also morphologically different from those found in adults and late regeneration. Specifically these arbors often branched one or more times between the common branch point and the endplate.

This excludes the possibility that the difference in length is due to remodelling of the axonal arbor or movement of the branch point. When individual axons innervate a muscle they will branch to form many regions of contact, some of which might be eliminated, even if that axon is ultimately the winner. However, the examples that we have described cannot be explained by this process because of they are different in length and morphology. First, there are converging sibling branches which are much longer than any of the pre-terminal branches observed in the adult and certainly further than the last heminode of Ranvier, which is only a few micrometres from the endplate. Second, many of the sibling branches (in fact at least one sibling branch in all four neonatal examples) have a sub-branch innervating a different endplate which diverged after the common branch point.

It is also not the case that sibling converging branches are always eliminated by a third converging branch from a different neuron. This is because in many of the instances of convergence, in both development and regeneration, the fluorescent neuron occupied the entire post-synaptic area, and so it is unlikely that the synapse was additionally innervated by a different motor neuron. This is an important observation because, although rare, the ability of sibling neurites to eliminate each other challenges current models of synapse elimination during development and regeneration.
We considered the possibility that converging axons belonging to the same neuron could both be preferentially eliminated in favor of terminals supplied by only one axon branch (see Results). This possibility could conclusively ruled out by examining end plates innervated by two different, differently colored axons (see for example, Kasthuri & Lichtman, 2003). This would represent a valuable extension to the present study and could provide elegant demonstration of the phenomenon of sibling neurite convergence, but the present data are sufficiently compelling to indicate the possibility is unlikely.

It is important that the competing branches can be distinguished, so that all of one branch's complement of active zones can be eliminated while those of the other branch are maintained. Hitherto, the only competition that has been considered is that between motor units, or between

axon branches within a motor unit that supply different muscle fibres (Brown, Jansen & Van Essen, 1976; Betz et al., 1979; Ribchester & Taxt, 1983; Ribchester, 1988a,b; Keller-Peck et al., 2001; Walsh & Lichtman, 2003; Kasthuri & Lichtman, 2003). These studies suggest that extensive loss of branches can occur within a motor unit even though other branches come to innervate other endplates exclusively. One interpretation of this is that all the branches of a single motor neuron are not equally competitive but another is that the competitive strength of branches and terminals changes as the motor unit becomes remodeled. Perhaps this dynamic in competitive strength extends, as we have shown here, to individual branches converging on the same endplate.

Synaptic competition at the NMJ is influenced by differing levels of activity, intracellular molecules or resources and by synaptic strength. It is easy to see how these differences could arise in axon branches belonging to different neurons, by each branch inheriting the properties of the neuron it belongs to. Indeed, Kasthuri and Lichtman (2003) demonstrated that there is a hierarchy within motor neurons, such that when two neurons compete at multiple synapses the same neuron will win at all these synapse. It is not clear whether differences between axon branches of the same neuron are sufficient for them to be distinguished by these forms of competition. Activity could be a differentiating factor for sibling neurites. While all adult sibling branches are thought to be active synchronously, the activity patterns in individual branches of a developing neonatal neuron have not been measured but could differ. Myelination of motor axons occurs gradually over the first two postnatal weeks, beginning in the nerve and eventually reaching the intramuscular and pre-terminal branches (Slater, 1982). Differences in the timing of myelination between sibling branches could lead to differences in the timing or the expression of activity, if not to the overall incidence. Sibling branches could also differ in the strength of their synapses. Differences in synaptic strengths have been hypothesised to influence the outcome of synapse elimination (Buffelli et al., 2003) and the same neuron can have synapses with differing synaptic strength (Trussell and Grinnell, 1985). However, if synapses belonging to the same neuron have the same range of properties as synapses belonging to different neurons, it is not clear why neuronal hierarchies would emerge, unless the within-unit variation was sufficient for competition but less than the variation between units.

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Figure Legends

Figure 1: A: Expected amount of sibling neurite convergence (SNC) in four different muscles. The expected amount of SNC increases with motor unit (MU) size. For a mouse fourth deep lumbrical (4DL) muscle with 250 muscle fibers and a neonatal motor unit size of 150, about 50 endplates should be innervated by converging sibling branches. In the other muscles, based on neonatal MU size data taken from the literature, we expect 51 (230), 75 (700) and 10 (122) instances of SNC (MU size) for mouse soleus, rat soleus and rat lumbrical respectively. B&C: control adult lumbrical endplates from a YFPH mouse without (B) and with (C) pre-terminal branching. For each endplate that was innervated by more than one branch, we measured the length of the branches (L1 and L2) from the common branch point to the synapse. Scale bar in B (also for C): 10 μm. D: Predicted amount of SNC with increasing variability on MU size. When the largest MU (LMU) is 5x larger than the smallest MU (SMU) more SNC is expected. E: Similarly the amount of SNC expected increases if muscle fibers have different probabilities of being innervated. max(MF) is the fiber with the largest innervation probability and min(MF) is the fiber with the smallest innervation probability. As the ratio increases so does the expected incidence of SNC. (Micrographs coloured magenta-green are provided in Supplementary Figure 1 for the benefit of red-green colour blind readers.)

Figure 2: Neonatal motor units in a YFPH LAL muscle (A-D) and YFP16 4DL muscle three days

after cutting the tibial nerve (E&F) muscles. A: A neonatal LAL motor unit innervating 15 muscle fibers. B: Traced motor unit in A. Branch order is represented by color, starting from dark red. The arrowhead in both indicates the entry point of the axon to the muscle. Three endplates (7, 8 and 10) are convergently innervated by sibling branches. According to Equation 1, two convergently innervated endplates were expected in this muscle C: Branching diagram of LAL motor unit in A&B. Full/half circles represent fully/partially occupied endplates respectively. The numbers on the left and the colors represent branching order. For example, an axon branch innervating an endplate on line 5 has branched 5 times from the point of entry. D: Magnified image of endplate 8. E: A neonatal 4DL motor unit innervating 214 muscle fibers. In the lumbrical muscle there were only a few equivocal examples of sibling neurite convergence, e.g. F. The putative convergently innervated endplate is marked with a '*' and the arrowheads show the two branches which may be innervating this endplate. (Micrographs coloured magenta-green are provided in Supplementary Figure 2 for the benefit of red-green colour blind readers). Scale bars in A (also applies to B), D and E: 100 μm, scale bar in F: 10 μm

Figure 3: Branches from multiple neurons fasciculate but branches from a single neuron do not. Examples of intramuscular nerves from YFPH LAL muscles where there is a single (A&B) or multiple (C&D) fluorescent axons. Axons from multiple neurons tend to fasciculate but axon branches from a single neuron do not. (Micrographs coloured magenta-green are provided in Supplementary Figure 3 for the benefit of red-green colour blind readers). Scale bar: 100 μm in A and 10 μm in B, 20 μm in C and D.

Figure 4: Convergence in regenerating motor units. A: Histograms of branch lengths in control adult, early and late regenerating adult YFPH deep lumbrical muscles. There are long converging branches (>50 μm) in early regeneration that don't appear in control muscles or late regeneration. Their absence from late regeneration suggests that one of the converging branches has been competitively eliminated. B-D: Three examples of convergently innervated endplates from early regeneration. White boxes show traces of the two converging branches from the common branch

point. Scale bars: 10 μ m (Micrographs coloured magenta-green are provided in Supplementary Figure 4 for the benefit of red-green colour blind readers) E: Scatter plot showing no correlation between the length of converging branches and the ratio of their diameters.

Figure 5: A: An entire adult YFPH 4DL motor unit from 14 days post-crush. B: Traced motor unit in A. Branch order is represented by color and the numbering corresponds to that in C. Dotted traces show uncertainty. Arrowheads indicate the point of entry of the nerve to the muscle. This unit innervates 53 endplates out of 165 and nine endplates should be convergently innervated. The number of doubly innervated endplates in this muscle is 8 out of which about half are longer than control pre-terminal branches. (Micrographs coloured magenta-green are provided in Supplementary Figure 5 for the benefit of red-green colour blind readers) Scale bars: 100 μ m C: Branching diagram of the motor unit. Each endplate is color-coded to show branch order. White endplates were insufficiently resolved to determine their innervation with certainty.

Supplementary Figure 1

Magenta-green version of Figure 1 for the benefit of red-green colour blind readers.

Supplementary Figure 2

Magenta-green version of Figure 2 for the benefit of red-green colour blind readers.

Supplementary Figure 3

Magenta-green version of Figure 3 for the benefit of red-green colour blind readers.

Supplementary Figure 4

Magenta-green version of Figure 4 for the benefit of red-green colour blind readers.

Supplementary Figure 5

Magenta-green version of Figure 5 for the benefit of red-green colour blind readers.

Table 1. Primary antibody

Table 2. The total potential innervation sites in developing LAL muscles includes only those in the region of the muscle that was innervated by the fluorescent axon. In the 4DL muscle, every muscle fibers was considered a potential innervation site. This and MU size were used to calculate the expected instances of SNC from Equation 1. These were almost always greater than the observed instances of SNC.

Table 3. For each of the three groups we show the number of singly innervated endplates and the number of doubly innervated endplates. Double innervated endplates are both those with sibling neurite convergence and those with pre-terminal branches. Unusual morphology refers to instances where one or both of the converging arbors have a sub-branch between the common branch point at the endplate that innervates a different muscle fiber.

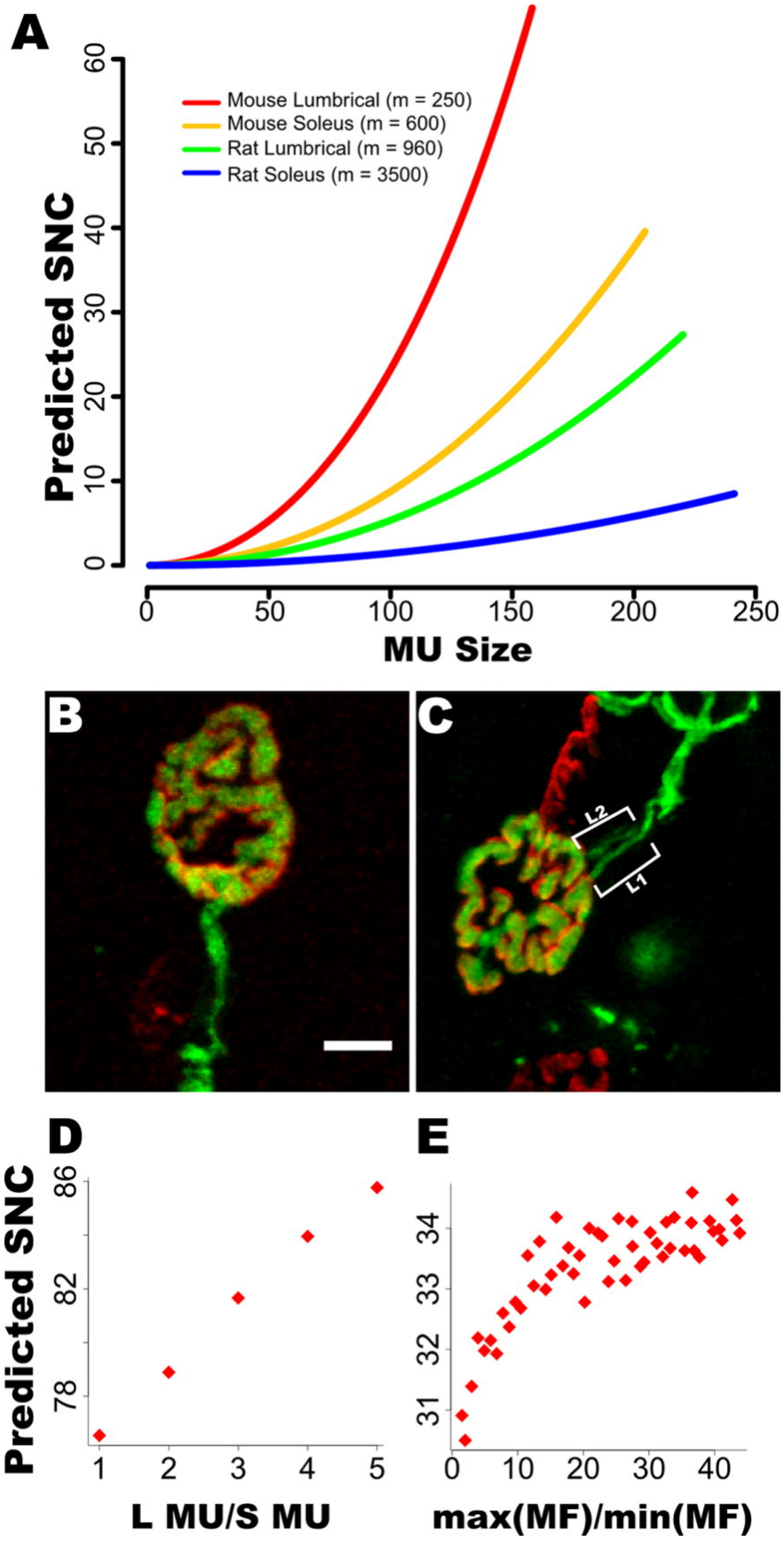
A
Predicted SNC
0
10
20
30
40
50
60
Mouse Lumbrical (m = 250)
Mouse Soleus (m = 600)
Rat Lumbrical (m = 960)
Rat Soleus (m = 3500)
0
50
100
150
200
250
MU Size
B
C
L2
L1
D
Predicted SNC
78
82
86
1
2
3
4
5
L MU/S MU
E
31
32
33
34
0
10
20
30
40
max(MF)/min(MF)

Figure 1

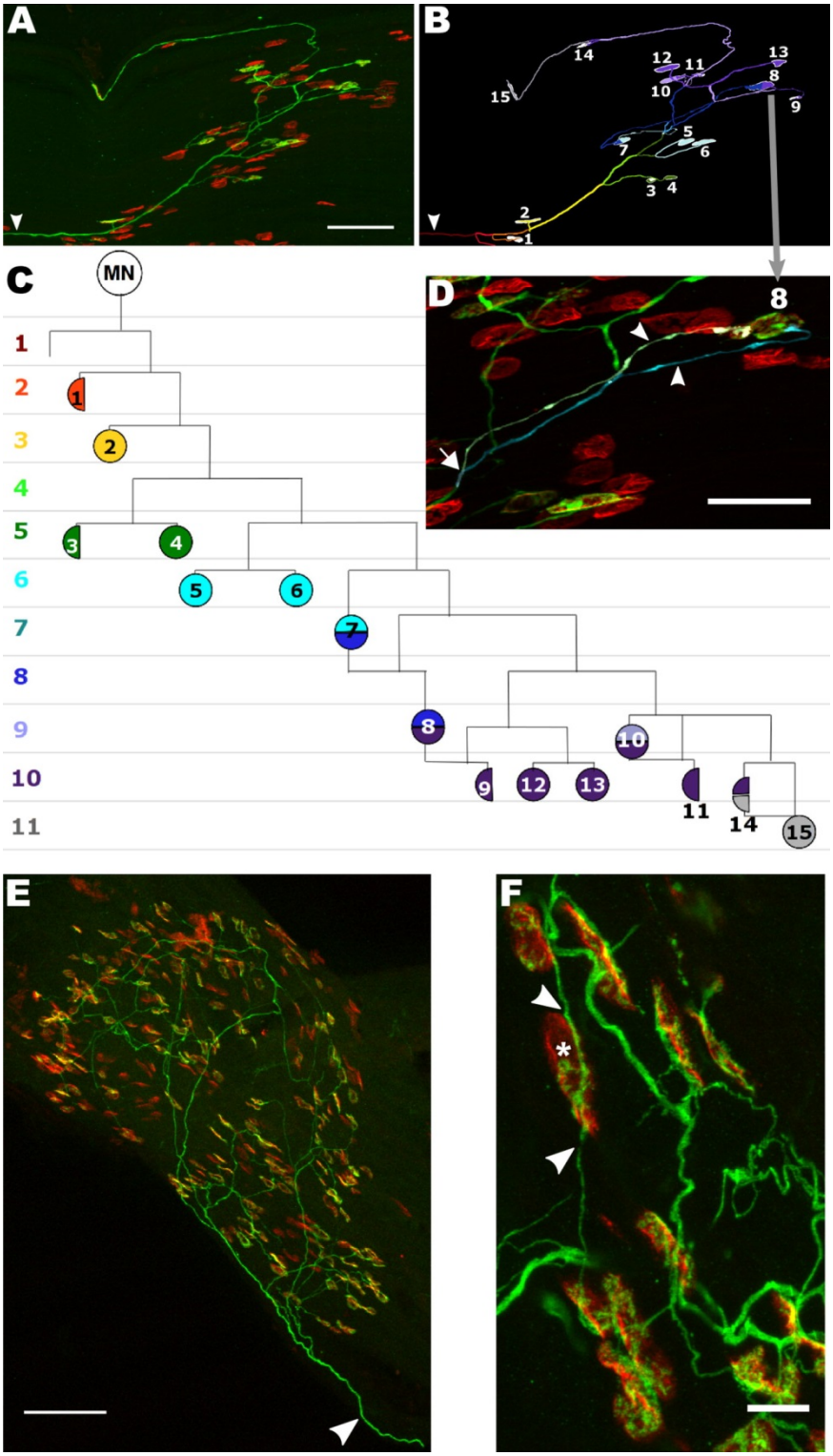

Figure 2

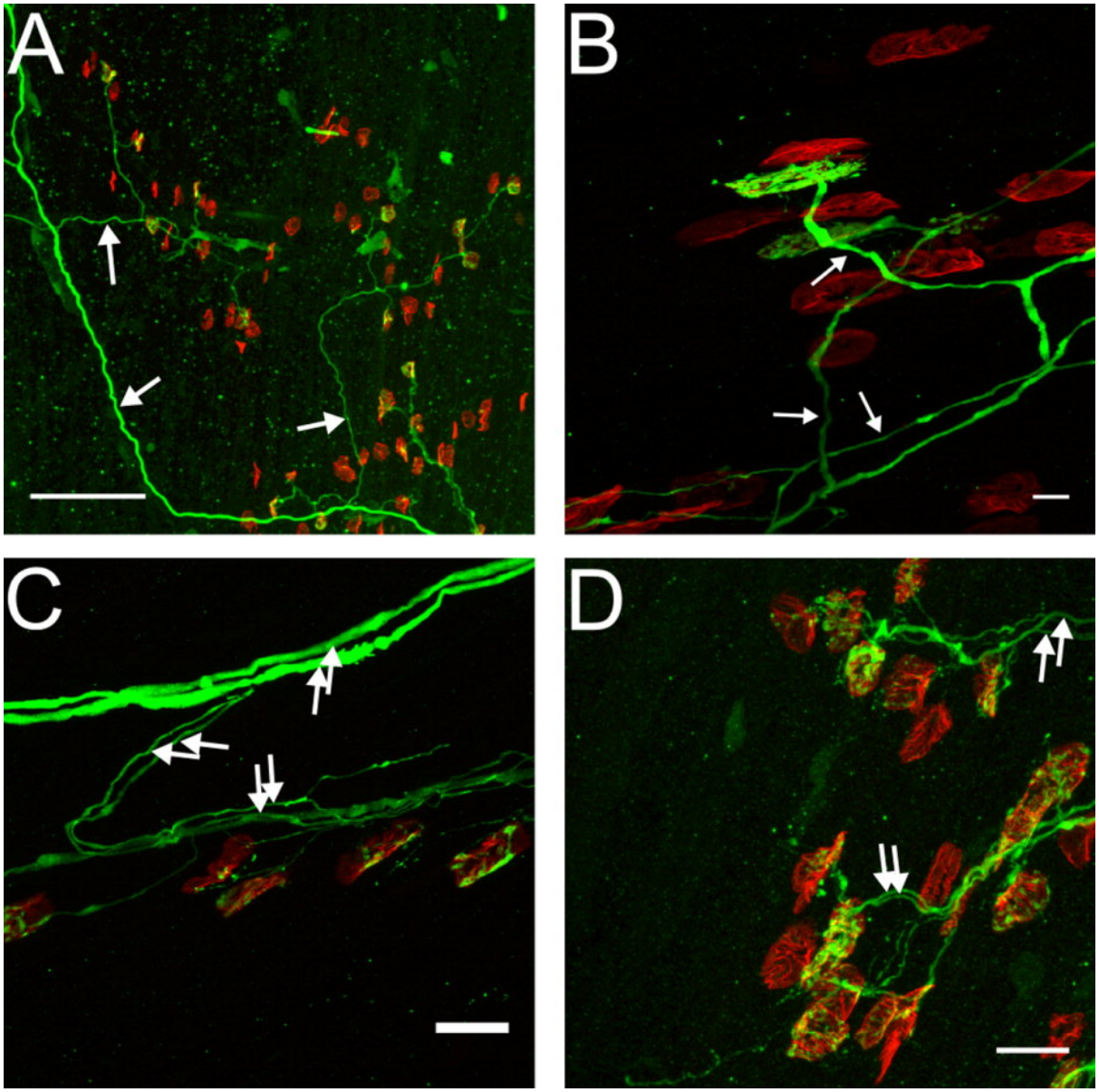

Figure 3

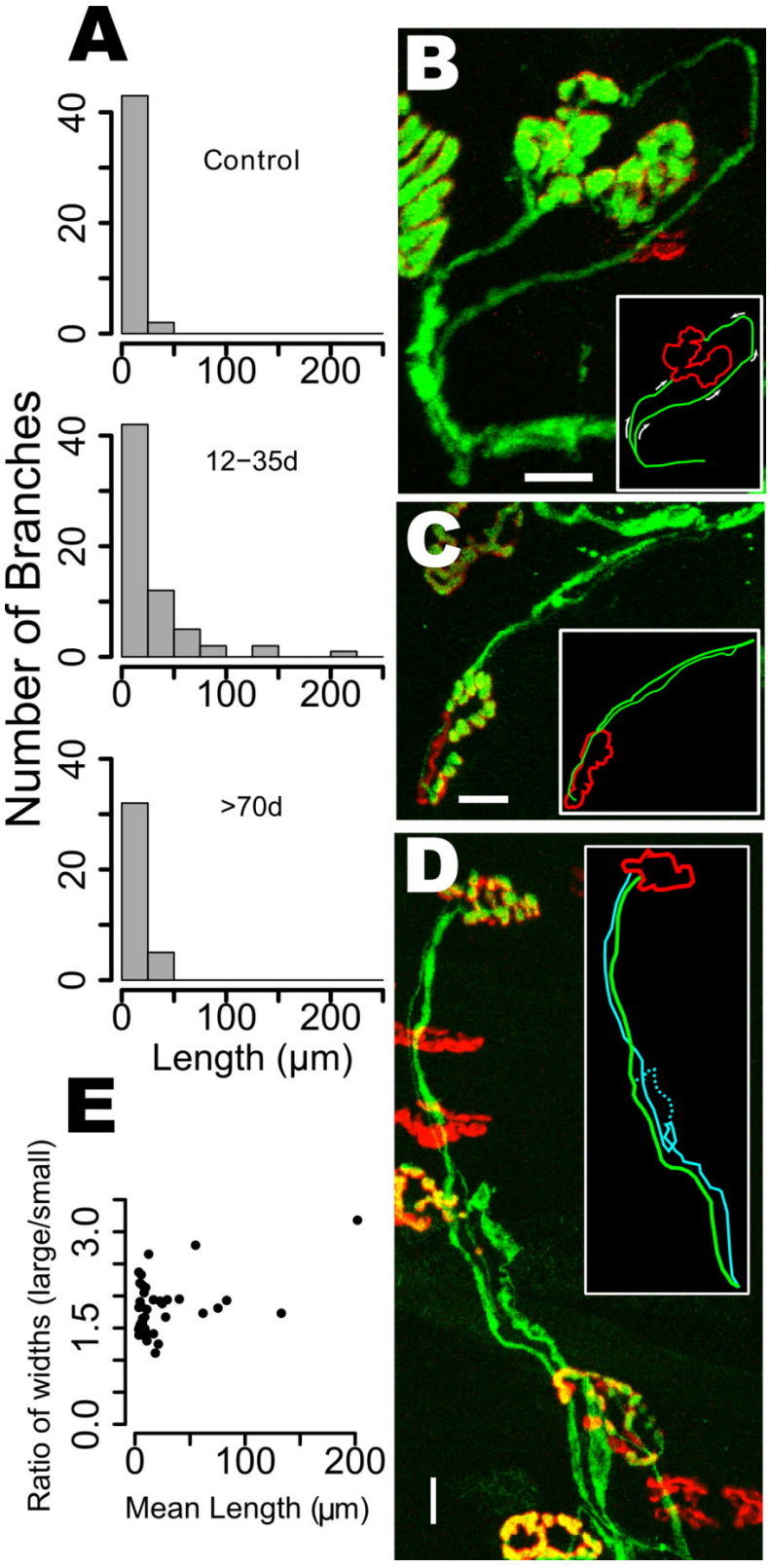
A
Control
12–35d
>70d
Number of Branches
0
20
40
0
100
200
Length (μm)
B
C
D
E
Ratio of widths (large/small)
0.0
1.5
3.0
Mean Length (μm)

Figure 4

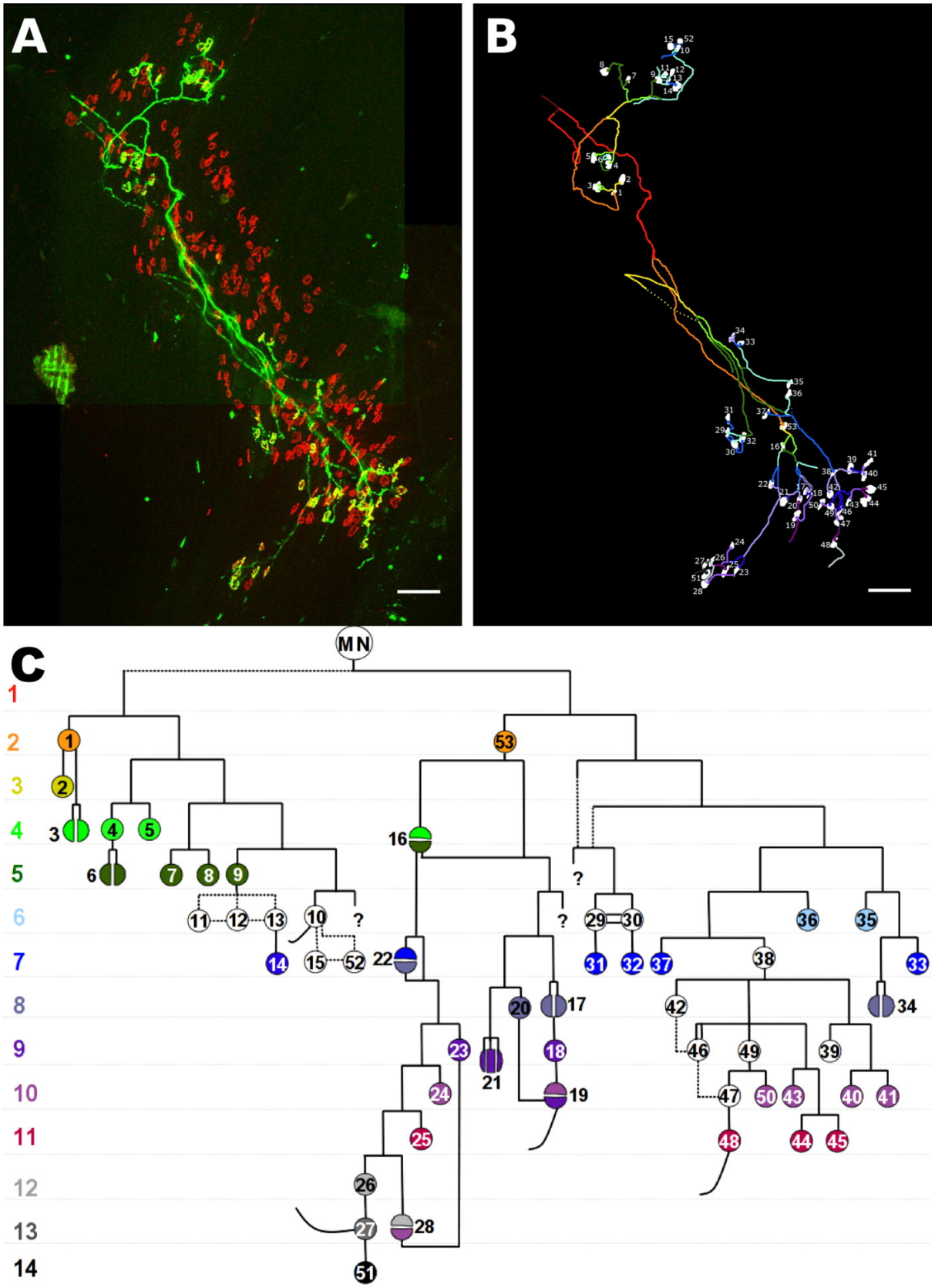

Figure 5

Table 1. Primary antibody

Antibody	Immunogen	Source/cat number	species	Dilution for IHC	Reference
Anti-GFP	Highly purified native GFP from Aequorea victoria	Millipore/AB3080	Rabbit polyclonal	1:1000	Schaefer et al. (2005)

Table 2. Data from single motor units in developing LAL and lumbrical muscles.

	Age	Total potential innervation sites	MU size	Expected instances of SNC	Observed instances of SNC
LAL	p5/p6	31	16	5	1
LAL	p5/p6	63	15	2	3
LAL	p5/p6	189	15	1	0
LAL	p5/p6	109	22	2	0
LAL	p6	224	61	9	0
4DL	p8	195	161	101	0
4DL	p8	302	214	105	<20

Table 3. Summary data from control early and late reinnervated lumbrical muscles.

	# synapses (#muscles/mice)	# single innervation	# double innervation	#double with unusual morphology	Mean length ± SD (min-max) μm
control	300 (6/5)	255 (85%)	45 (15%)	0 (0%)	8.6 ± 6.6 (2-30)
early	525 (9/7)	457 (87%)	68 (13%)	15 (2.8%)	27.5 ± 35.2 (2-228)
late	327 (8/7)	290 (89%)	37 (11%)	2 (0.6%)	12 ± 10 (3-47)