Branch-Specific Microtubule Destabilization Mediates Axon Branch Loss during Neuromuscular Synapse Elimination

Highlights
- During synapse elimination, retreating axon branches dismantle their microtubules
- Microtubules are destabilized due to branch-specific severing
- Microtubule stabilization delays axon branch removal during synapse elimination
- The disease-associated microtubule severing protein spastin mediates microtubule loss

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In Brief
Brill, Kleele, Ruschkies et al. show that microtubule destabilization mediates dismantling of retreating axon branches and excludes them from organelle delivery by axonal transport. This mechanism is branch specific and involves the neurodegeneration-associated microtubule severing protein spastin.
Branch-Specific Microtubule Destabilization Mediates Axon Branch Loss during Neuromuscular Synapse Elimination

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SUMMARY

Developmental axon remodeling is characterized by the selective removal of branches from axon arbors. The mechanisms that underlie such branch loss are largely unknown. Additionally, how neuronal resources are specifically assigned to the branches of remodeling arbors is not understood. Here we show that axon branch loss at the developing mouse neuromuscular junction is mediated by branch-specific microtubule severing, which results in local disassembly of the microtubule cytoskeleton and loss of axonal transport in branches that will subsequently dismantle. Accordingly, pharmacological microtubule stabilization delays neuromuscular synapse elimination. This branch-specific disassembly of the cytoskeleton appears to be mediated by the microtubule-severing enzyme spastin, which is dysfunctional in some forms of upper motor neuron disease. Our results demonstrate a physiological role for a neurodegeneration-associated modulator of the cytoskeleton, reveal unexpected cell biology of branch-specific axon plasticity and underscore the mechanistic similarities of axon loss in development and disease.

INTRODUCTION

Many developing neurons initially form exuberant axonal projections. Postnatal pruning then selectively removes redundant or anatomically inappropriate axon branches (Kano and Hashimoto, 2009; Lichtman and Colman, 2000). Such pruning comes in diverse manifestations that differ in morphology and extent, as well as in driving force, which in some cases are stereotypical molecular cues while in others are activity-dependent competition. Still, in this variety of settings—ranging from invertebrates to mammals and the peripheral to the central nervous system—one property appears to be conserved: individual branches of an axonal arbor are selectively pruned back to their branch point while leaving the remainder of the axon intact or even strengthened (Luo and O’Leary, 2005; Riccomagno and Kolodkin, 2015; Schuldiner and Yaron, 2015). Currently, our understanding of such branch-specific remodeling is sketchy, even though its significance extends beyond development, as pruning and neurodegenerative “die-back” might share a core set of cell biological mechanisms and molecular mediators (Coleman and Perry, 2002; Yaron and Schuldiner, 2016). For example, many forms of axon pruning and degeneration are characterized by disruption of the cytoskeleton (d’Ydevalle et al., 2011; Kurup et al., 2015; Massaro et al., 2009; Watts et al., 2003). Microtubules seem especially critical in disease settings, as they not only impart shape, but also mediate transport and signaling (Conde and Cáceres, 2009; Fletcher and Mullins, 2010). Moreover, numerous post-translational modifications and associated proteins can regulate microtubule turnover and might hence determine local axon stability (Janke and Bulinski, 2011; Janke and Kneussel, 2010). Indeed, a number of pharmacological and genetic perturbations of microtubules manifest in axonal die-back (Niwa et al., 2013; Röytä and Raine, 1986; Solowska and Baas, 2015). In converse, microtubule stabilization has been hailed as a potential therapy for various neurologic conditions (Cartelli et al., 2013; Das and Miller, 2012; Ertürk et al.,
Beyond pathological settings, however, the role of the microtubular cytoskeleton in local axon loss is more controversial. While some previous studies have found microtubule loss in developmental pruning (Bishop et al., 2004; Maor-Nof et al., 2013; Schuldiner and Yaron, 2015; Watts et al., 2003; Williams and Truman, 2005), other influential models surmise a critical role for intact microtubules and transport in the pruning process (Barber and Lichtman, 1999; Morris and Hollenbeck, 1993; Riley, 1981). For example, it has been proposed that organelles are “evacuated” by fast retrograde transport from dismantling axon branches, inconsistent with the notion that microtubule loss would be an early and causative step in axon loss (Liu et al., 2010). Moreover, whether microtubule disassembly in a remodeling axon arbor is locally confined, and which factors might mediate it, remains elusive. Overall, experimental interrogation of the relationship between cytoskeletal changes and axon remodeling is challenging, as few forms of developmental pruning are accessible to branch-specific assays that allow concomitant assessment of cytoskeletal and axon dynamics.

In this work, we take advantage of the developing mammalian neuromuscular junction (NMJ), a prime vertebrate model of branch-specific axon pruning, to address the mechanisms that mediate axon branch loss (Lichtman and Colman, 2000). During prenatal neuromuscular synaptogenesis, up to ten branches of different motor axons converge on the same synaptic site on a muscle fiber (Tapia et al., 2012). Subsequently (in the mouse during the first two postnatal weeks), all but one of these branches are removed by activity-dependent synapse elimination. During this process, a motor neuron typically loses the majority of its initial branches while simultaneously stabilizing and expanding those that remain. We have previously shown that dismantling axon branches eventually sheds fragments that are digested by glial cells (Bishop et al., 2004; Song et al., 2006). This final dismantling to establish single innervation is preceded by a phase of ongoing competition, where one of the synaptic inputs expands at the expense of the other (Walsh and Lichtman, 2003). While this competition process is not necessarily monotonic, as it is affected by the outcome of other remote synaptic competitions that the involved motor neurons engage in (Kashturri and Lichtman, 2003), the synaptic territory of a branch can still be used as a surrogate to predict likely outcome (Walsh and Lichtman, 2003). Notably, expanding and shrinking axon branches of all stages of competition coexist alongside the developing motor unit, intermingled in an apparently stochastic distribution (Keller-Peck et al., 2001). Despite this relatively comprehensive understanding of the general ruleset of this synapse remodeling process and its significance for the final neuro-motor circuit, the cell biological events that precede the dismantling of single branches of a developing motor unit have remained obscure. We now elucidate the mechanisms that underlie axon branch dismantling by monitoring cytoskeletal function and stability in axon branches during different stages of synapse elimination using tools that we have previously developed (Kleele et al., 2014; Misgeld et al., 2007; Sorbara et al., 2014). Our results reveal loss of microtubules as a driving force of branch-specific axon loss during synapse elimination, in part mediated by the neurodegeneration-associated microtubule-severing protein spastin.

RESULTS

Organelle Evacuation Does Not Precede Initiation of Axon Branch Loss

We first explored whether there is a phase of organelle evacuation from retreating branches as proposed in the original model of neuromuscular synapse elimination (Riley, 1981) and since demonstrated for remodeling Drosophila NMJs (Liu et al., 2010) or whether the opposite prediction, namely of absent transport in dismantling axon branches, holds true. To this end, we devised a method of sequential photo-bleaching in postnatal days 7–13 (P7–P13) nerve-muscle explants from Thy1-XFP mice (Thy1-YFP-16 or Thy1-CFP-5; Feng et al., 2000) to determine the fraction of a neuromuscular postsynaptic site that a given motor axon branch occupied (Figures 1A–1E; Brill et al., 2011; Turney and Lichtman, 2012). This territory can serve as a predictor of the outcome of synaptic competition (Walsh and Lichtman, 2003). We then measured the relationship of net delivery (i.e., anterograde minus retrograde flux) of fluorescently labeled mitochondria (Thy1-mitoCFP-K; Misgeld et al., 2007) to the synaptic territories held by a pair of competing branches (Figure 1F; Figures S1A and S1B). Notably, apparently “shrinking” axon branches (i.e., those that held 40% or less of a synapse’s area) showed neither mitochondrial delivery nor evacuation (net transport not significantly different from 0; p = 0.12, Wilcoxon signed rank test; n = 53 axons/53 mice). In contrast, “undecided” (centered on 50% territory) and apparently “growing” (>60%) axon branches received a stable net number of mitochondria (net transport for 41%–60% branches did not differ significantly from branches that innervated 61%–80%, 81%–99%, or 100% of a synapse; p = 0.844, Kruskal-Wallis test; n = 41 axon/41 mice; 61%–80%, n = 30/30; 81%–99%, n = 24/24; and 100%, n = 128/66). In addition to mitochondria, also the transport of peroxisomes was selectively abolished in retreating branches (Figures S1C and S1D; measured in Thy1-PeroxyYFP mice; Sorbara et al., 2014). These results argue against an “evacuation” model of axon dismantling for the mouse NMJ (Liu et al., 2010; Riley, 1981) and further suggest the remodeling of microtubular transport tracts as a possible cause of these branch-specific transport deficits, as transport of at least two organelles in both directions was affected (Figure S1).

The Microtubular Cytoskeleton Is Specifically Dismantled in Terminal Axon Branches before They Retreat

To characterize the status of the microtubular cytoskeleton with single-branch precision, we determined synaptic territories by sequential photo-bleaching and then processed NMJs for quantitative immunostainings of βIII-tubulin (normalized to transgenically expressed YFP; see Supplemental Experimental Procedures for details). In retreating axon branches, tubulin levels dropped as territory shrunk, with retraction bulbs showing a substantial (52%) reduction compared to synapses in the midst of competition (Figure 1G; 0% versus 41%–60%,
p < 0.0001, Mann-Whitney test; 0%, n = 55 axons/9 mice; 41%–60%, n = 25/7), while in consolidated axons (>60%), tubulin levels further increased (by 27%; Figure 1G; 41%–60% versus 100%, p = 0.05, Mann-Whitney test; 100%, n = 54 axons/9 mice). Comparable results were obtained with further antibodies directed against βIII-tubulin and α-tubulin, while neurofilaments were unaffected (Figure S2), suggesting that microtubules were specifically lost. Indeed, when we took advantage of mice that express a fluorescently labeled plus-end binding protein (Figure 2; Thy1-EB3-YFP; Kleele et al., 2014), we found a substantial increase in the density of EB3 “comets” in axon branches with territory of 40% or less (Figure 2A; Movie S1; significance was already reached in axons with 21%–40% territory versus 41%–60%, p < 0.01, unpaired t test; 21%–40%, n = 16 axons/11 mice; 41%–60%, n = 14/8). This assay highlights growing microtubule tips that are capped by end-binding proteins (such as EB3). It allows visualizing an aspect of the dynamics of the microtubule cytoskeleton and—when combined with a measure of microtubule mass, such as tubulin staining—can be used as a surrogate measure of microtubule length (Perez et al., 1999; Stepanova et al., 2003; Baas et al., 2016). Indeed, the ratio of EB3 over βIII-tubulin increased dramatically as axons shrank to less than 40% of territory. Thus, a highly destabilized and fragmented microtubular cytoskeleton exists in such apparently shrinking inputs, while in apparently growing axon branches, the cytoskeleton appears rather stable (Figure 2B). Importantly, the observed changes are not the result of a general developmental expansion and stabilization of the microtubule cytoskeleton from which retreating branches were excluded, as we found for all measures (mitochondrial transport, tubulin immunostaining, and EB3 density) that axon branches of all territory bins at P7/P8 exhibited the same characteristics as their counterparts two days later (P9/P10), i.e., across a developmental time window typically required for a synapse to transition from double to single innervation (Walsh and Lichtman, 2003). For example (Figure S3), this developmental profile indicates that retraction bulbs at P9/P10 contain less tubulin than the two branches serving a doubly innervated synapse did at P7/P8, i.e., those axon branches of which one will convert to a retraction bulb in ~62% of cases within the next two days (percentage of doubly innervated synapses at P7/P8: 25.2% ± 3.4% versus P9/P10: 9.7% ± 1.3%, n ≥ 6 mice). To corroborate that reduced tubulin staining indeed corresponded to actual loss of microtubules...
and concurrently validate the bleaching approach, we reconstructed a subset of bleached NMJs by correlated electron microscopy (Figures 3A and 3B; Movie S2; Bishop et al., 2004). This approach confirmed a profound loss of microtubules in apparently shrinking branches (Figures 3C–3F), following a quantitative relationship similar to the one seen previously by immunohistochemistry (Figure 3G, cf. Figure 1G), even though the latter appears to also label a base level of tubulin not detectable by electron microscopy.

The fact that during NMJ synapse elimination single branches in an axonal arbor are dismantled, while sister branches are preserved and even strengthened (Colman et al., 1997; Keller-Peck et al., 2001), suggests that microtubule destabilization must be a precisely tuned and compartmentalized process. Indeed, when we measured EB3 comet density in proximal and distal positions along axon branches tipped by retraction bulbs, we found the same average density of EB3 comets (Figures 2C and 2D), while the stem axon of a retraction bulb showed the same level of EB3 comets as stem axons of branches that had established single innervation (Figures 2E and 2F). Thus, microtubular destabilization is specific to and compartmentalized within retreating branches.

**Pharmacological Stabilization of Microtubules Delays Synapse Elimination**

Our data suggest that branch-specific microtubule destabilization represents a seminal step in branch dismantling. This predicts that stabilizing the microtubular cytoskeleton should delay synapse elimination. Notably, stabilizing microtubules using epothilone, a readily bioavailable alternative to paclitaxel, is currently being explored as an approach to preserve axons after trauma (Ruschel et al., 2015) and during neurodegeneration (Cartelli et al., 2013; Zhang et al., 2012). Treatment of mice with a single injection of epothilone B on P5 caused a significant delay in synapse elimination (Figure 4A) with multiple innervation present at least until P21 (Figure 4B). We confirmed that epothilone did not delay overall neuromuscular development (Figures S4A–S4D), while it increased tubulin content and reduced EB3 comet density in both retreating and established "winner" axons in a dose-dependent manner (Figures 4C–4E; Figures S5A–S5C). Finally, stabilizing microtubules increased mitochondrial transport in retreating axon branches. This effect was relatively less pronounced in the unbranched stem of axons found in the intercostal nerve and in terminal axon branches that have established single innervation (Figures S5D and S5E).

**Spastin Destabilizes Microtubules in Retreating Axons and Promotes Synapse Elimination**

The fact that global stabilization of microtubules delayed branch removal raised the question of which mechanism might mediate specific destabilization of the microtubular cytoskeleton in apparently shrinking branches. A number of post-translational modifications have been linked to microtubule turnover (Janke and Bulinski, 2011; Janke and Kneussel, 2010) and hence might contribute to the molecular changes that accompany branch-specific microtubule destabilization. Using immunostainings for post-translational tubulin modifications in retreating axons and concurrently validate the bleaching approach, we reconstructed a subset of bleached NMJs by correlated electron microscopy (Figures 3A and 3B; Movie S2; Bishop et al., 2004). This approach confirmed a profound loss of microtubules in apparently shrinking branches (Figures 3C–3F), following a quantitative relationship similar to the one seen previously by immunohistochemistry (Figure 3G, cf. Figure 1G), even though the latter appears to also label a base level of tubulin not detectable by electron microscopy.

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to account for the diminishing overall tubulin levels; Figures 5A–5H), we found the expected change in glutamylation/tyrosination ratio characteristic of a hyper-dynamic microtubular cytoskeleton, as well as a selective drop of polyglutamylation, which occurred once an axon’s territory had fallen below 40% (Figure 5I). There was no evidence for selective deacetylation (Figures 5E and 5F), which has previously been linked to microtubule destabilization (d’Ydewalle et al., 2011; Matsuyama et al., 2002). These data suggested that tubulin deacetylasers are not causal in the branch-specific dismantling process but that other microtubule-modifying enzymes, some of which are sensitive to post-translational modifications (Lacroix et al., 2010; Valenstein and Roll-Mecak, 2016), might mediate the local destabilizing effects on the microtubule cytoskeleton. For example, microtubule-severing enzymes, such as spastin, would be expected to cause an increase in the number of microtubule ends, while at the same time potentially inducing the loss of microtubule mass (Baas et al., 2016). Spastin especially has been implicated, on the one hand, in regulating axon branching and synapse development (Trotta et al., 2004; Wood et al., 2006; Yu et al., 2008), as well as, on the other hand, degeneration of motor tracts in hereditary spastic paraparesis (Solowska and Baas, 2015). Notably, the most affected axon projection in this disease, the corticospinal tract, is also a site of axon branch remodeling (Stanfield et al., 1982).

To test the involvement of spastin in synapse elimination and the microtubule destabilization that accompanies it, we generated a new spastin knockout (KO) mouse via the “knockout-first” approach to characterize synapse elimination, as well as axonal microtubule content and turnover (Figure 6). In contrast to other species, where knockdowns of spastin results in motor axon defects (Trotta et al., 2004; Wood et al., 2006), in mice, spastin deletion does not result in an obvious phenotype in young animals (Tarrade et al., 2006; Kasher et al., 2009). In line with these previous murine loss-of-function mutants of spastin, our spastin knockout mice also showed no detectable neuromuscular phenotype at birth (such as abnormal...
sprouting; Figure S6) or during postnatal maturation of neuromuscular junctions (Figures S4E–S4H). Strikingly, synapse elimination was substantially delayed in these mutants (Figures 7A and 7B). When we measured the speed with which retraction bulbs shortened in spastin-mutant mice (Bishop et al., 2004), we found a significant reduction compared to the wild-type situation (Figures 7C–7E). As expected, βIII-tubulin content was increased in retreating, as well as stabilized, axon branches, while an increase in EB3 comet density commensurate to an increased microtubule mass was only evident in stabilized branches at singly innervated NMJs, but not in retraction bulbs (Figures 7F and 7G). Hence, global suppression of spastin gene expression selectively—albeit only partially—reduced microtubule degradation as indicated by the ratio of comet density and tubulin content (Figure 7H). As spastin is widely expressed (Ma et al., 2006), we ensured the site of action of spastin by using a ChAT-Cre mouse line (cre recombinase driven by the choline acetyltransferase [ChAT] promoter; Figure 7I) to generate a cell-type-specific spastin knockout mouse lacking spastin gene expression selectively in cholinergic neurons. Again, when analyzing the degree of multiple innervation, we found that loss of spastin gene expression in motor neurons only led to a significant delay in synapse elimination from P7 to P21 (Figure 7J). Similarly, when we intraven-

**DISCUSSION**

Here we demonstrate that during NMJ synapse elimination, branch-specific destabilization of the microtubular cytoskeleton parallels failure of organelle delivery and induces axon branch dismantling. This effect is mediated in part by the microtubule-severing protein spastin. Our results provide insight into a hitherto largely unresolved question related to neuronal remodeling and reveal new parallels between developmental plasticity and neurodegeneration. Moreover, while dendritic sub-compartments play an important role in many models of circuit plasticity (Cichon and Gan, 2015; Kanamori et al., 2013; Losonczy et al., 2008; Yang et al., 2014), axon branches are less frequently discussed in this context (Riccomagno and Kolodkin, 2015).

Notably, our results establish that axons are capable of branch-specific remodeling of the microtubular cytoskeleton, which results in changed resource allocation by axonal transport. Here our work has two important implications: (1) early microtubule degradation in branches destined for dismantling argues against the necessity of evacuation by axonal transport (Liu et al., 2010; Morris and Hullenbeck, 1993; Riley, 1981) but...
is consistent with our previous work that suggested local piecemeal shedding and degradation of dismantling axon branches and their content (Bishop et al., 2004; Song et al., 2008) and (2) within an axonal arbor, organelle transport is matched to the down-stream target size. This holds true even at the level of terminal axon branches, where anterograde transport corresponds to synaptic territory (Figure S1B), which in turn corresponds to the degree of local microtubule destabilization (Figures 1G and 2A). This implies that axonal transport in developing axonal arbors is carefully regulated and capable of following alterations in synaptic target size (Aletta and Goldberg, 1982). Thus, axonal transport might provide a cell biological correlate for the resource allocation that previous modeling and experimental work have predicted as an important outcome determinant of synapse elimination (Barber and Lichtman, 1999; Kasthuri and Lichtman, 2003).

In any case, microtubule degradation likely not only affects transport into dismantling branches, but could directly contribute to shrinking and atrophy of the axon branch (Bernstein and Lichtman, 1999), as microtubules can directly impart shape, in part, by cross-talk to the actin cytoskeleton (Conde and Caceres, 2009; Fletcher and Mullins, 2010). Indeed, our previous work on the final phase of neuromuscular axon remodeling has shown that the disassembly of the tip of a retreating axon branch by “axosome shedding” correlates with general cytoskeletal disruptions (Bishop et al., 2004). However, an early instructive role of local microtubule dismantling was not anticipated. Moreover, the involvement of the neurodegeneration-associated protein spastin in this setting is new. Indeed, while spastin can clearly sever microtubules in vitro, and its disruption can affect long axon tracts—including motor neurons—in human disease and related models (Wood et al., 2006; Butler et al., 2010; Fassier et al., 2013), how spastin acts at its putative site of action, the microtubular cytoskeleton, remains somewhat controversial (Solowska and Baas, 2015). Our work clarifies this for mammalian axons in vivo, as deletion of spastin manifests in our assays of microtubule content and fragmentation as a (weaker) version of microtubule stabilization (cf. Trotta et al., 2004). This effect appears to primarily affect retreating branches and, to only a lesser extent, winner branches that have established single innervation (cf. Figure 7H versus Figure S5C, where a dose of 0.25 \( \mu \)g/\( \mu \)L epothilone displayed a similar action profile as loss of spastin). This suggests that either spastin acts specifically in retreating branches (e.g., because of selective activation of spastin or

Figure 5. Post-translational Modifications of Microtubules in Axon Branches of Different Competition State

(A)–(I) show stainings for post-translationally modified microtubules (red) superimposed on \( \beta \)-III-tubulin (white). Quantification of post-translational modifications normalized to \( \beta \)-III-tubulin in bulb-tipped retreating axon branches (rebu) and in singly innervating axon branches (sin; B: \( n \geq 38 \) axons from 4 mice per group; D: \( n \geq 41, 6 \); F: \( n \geq 57, 4 \); H: \( n \geq 41, 5 \)). Levels of polyglutamylated tubulin (normalized to \( \beta \)-III-tubulin) versus synaptic territory of competing axon branches (\( n \geq 161 \) axons from 10 mice) (I). The monochrome left-hand panels are adjusted with non-linear gamma to enhance visibility of the thin retreating axons; in the merged right-hand panels, both channels are linearly adjusted (A, C, E, F, and G). Scale bars, 5 \( \mu \)m throughout. Data are mean + SEM. Mann-Whitney test was used to determine significance: ***p < 0.001; **p < 0.01; n.s. p ≥ 0.05.
locally constrained microtubule modifications/microtubule binding proteins that alter susceptibility to severing; Lacroix et al., 2010; Valenstein and Roll-Mecak, 2016) or compensation by other microtubule severing proteins (e.g., katanin; Havlicek et al., 2014) might be more efficient in patent winner branches.

In line with this preservation of a cytoskeletal scaffold that acts as transport tracts, both epothilone treatment and, to a lesser extent, spastin deletion could partially restore organelle transport to retraction bulb-tipped axon branches (epothilone; see Figures S5D and S5E; spastinKO anterograde: 0.1 ± 0.1 in KO versus 0.0 ± 0.0; retrograde: 0.3 ± 0.2 in KO versus 0.1 ± 0.1 mitochondria/hr in control, n = 9 axons/9 mice).

It might seem paradoxical that, in our setting, spastin’s action apparently mediates branch loss and suppresses axonal transport, while spastin is known to be required for axon branching in vitro and in non-mammalian species (Wood et al., 2006; Yu et al., 2008) and also to promote axonal transport in vitro (Kasher et al., 2009). In our spastin mutants (as in previously reported mouse mutants; Tarrade et al., 2006; Kasher et al., 2009), we could not detect a phenotype that would indicate a severe axon outgrowth disturbance (as reported in spastin knockdowns using morpholinos in zebrafish; Wood et al., 2006), potentially suggesting differential species-specific compensation. At the same time, the fact that, in different setting, spastin might affect neuronal geometry and organelle dynamics differentially is perhaps not so surprising given that the basic enzymatic activity of spastin is simply to sever microtubules. In some settings (e.g., during axon elaboration), local severing is needed to seed new microtubules and allow collateral formation; in others, it might be required for dismantling. Similarly, depending on the state of the microtubule cytoskeleton, severing might improve or diminish the ability to support organelle translocation. Indeed, the double-edged nature of changing the stability of microtubules for neuronal cell biology is well appreciated—after all, the main side effect of drugs that stabilize microtubules is neuropathy (Röyttä and Raine, 1986). At the same time, such drugs might be useful in promoting axon regrowth and survival (Ruschel et al., 2015).

Interestingly, the interplay of transport, cytoskeletal destabilization, and spastin in synaptic remodeling and axon degeneration that we implicate here in neuromuscular synapse elimination could not detect a phenotype that would indicate a severe axon outgrowth disturbance (as reported in spastin knockdowns using morpholinos in zebrafish; Wood et al., 2006), potentially suggesting differential species-specific compensation. At the same time, the fact that, in different setting, spastin might affect neuronal geometry and organelle dynamics differentially is perhaps not so surprising given that the basic enzymatic activity of spastin is simply to sever microtubules. In some settings (e.g., during axon elaboration), local severing is needed to seed new microtubules and allow collateral formation; in others, it might be required for dismantling. Similarly, depending on the state of the microtubule cytoskeleton, severing might improve or diminish the ability to support organelle translocation. Indeed, the double-edged nature of changing the stability of microtubules for neuronal cell biology is well appreciated—after all, the main side effect of drugs that stabilize microtubules is neuropathy (Röyttä and Raine, 1986). At the same time, such drugs might be useful in promoting axon regrowth and survival (Ruschel et al., 2015).

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has precedent: recently, it was shown that synapse disassembly during *C. elegans* development, albeit in the absence of gross morphological changes in neuritic arbors, depends on a similar mechanism to the one described here (Kurup et al., 2015). Moreover, katanin-like proteins are involved in developmental severing of dendrites in flies (Lee et al., 2009). Also, in vitro, some of the acute toxicity of the Alzheimer disease-associated Aβ peptide might be due to spastin-mediated disassembly of microtubules (Zempel et al., 2013). However, in contrast to Zempel et al. (2013), we did not observe a spike in polyglutamyalted microtubules (compared to the overall tubulin stain) at early stages of synapse elimination, but rather a late decay (in axon branches with <20% synaptic territory; Figure 5I), suggesting that the relative drop in this microtubule species is not necessarily indicative of an instructive role of this post-translational modification. Rather, it could be the result of a shift in local turnover to which, in addition to local severing activity, loss of selective stabilizers or altered function of the enzymatic machinery that imposes this post-translational modification could contribute. In line with the interpretation that the loss of polyglutamyalted tubulin in losing axon branches is in part due to spastin, we observed a...
massive increase in this post-translational modification in spastin knockout mice, especially in retracting axon branches (8.5 ± 0.23-fold increase in retraction bulbs, p < 0.0001 Mann-Whitney test; KO, n ≥ 34 axons/4 mice; WT, n ≥ 18/3; 3.8 ± 0.1-fold increase in singly innervating axon branches, p < 0.0001, Mann-Whitney test; KO, n ≥ 86 axons/4 mice; WT, n ≥ 47/3).

Together, our new work and the current literature suggests that the local microtubule-severing mechanism during axonal remodeling, documented here for mammalian axon development, might in its core be evolutionarily conserved and reactivated during disease (Yaron and Schuldiner, 2016; cf. Sekar et al., 2016, for another recent example). At the same time, a role for microtubule destabilization in physiological pruning processes provides a caveat for ongoing efforts to harness microtubule stabilization for therapy (e.g., using epothilone), especially after acute insults, such as spinal cord injury, where axonal remodeling and synapse elimination might be important mechanisms of compensatory plasticity (Raineteau and Schwab 2001; Bareyre et al., 2004).

**EXPERIMENTAL PROCEDURES**

**Animals**

Thy1-YPF-16 mice (cytoplasmic YFP expression in all motor neurons; Jackson Laboratory #003709, Tg(Thy1-YPF)16Jrs/J; Feng et al., 2000) were used to map synaptic territory of individual axons by sequential photo-bleaching for quantitative immunohistochemistry, electron microscopy, and analysis of the number of poly-innervated NMJs following epothilone B injections. To study axonal transport and visualize synaptic territory simultaneously, we used double-transgenic Thy1-YPF-16 × Thy1-mitoCFP-K mice (CFP is selectively expressed in neuronal mitochondria; Miegoy et al., 2007). We measured transport of peroxisomes and synaptic occupancy in Thy1-PeroxisomePF-339 or Thy1-PeroxisomePF-376 (labeling of neuronal peroxisomes; Sorbara et al., 2014) crossed to Thy1-CFP-5 or Thy1-OPF-3 mice (expression of CFP or OPF in all motor neurons; Feng et al., 2000; Brill et al., 2011). EB3 comet density and synaptic territory were assessed in double-transgenic Thy1-EB3-YFP-J045 (specific labeling of growing plus-end tips of microtubules; Kleele et al., 2014). To generate a knockout-first allele (Testa et al., 2004) of spastin in mice, we used the targeting vector (PG00198_Z_2_G10, project CSD77496) generated by the trans-NIH Knock-Out Project and obtained from the KOMP Repository used the targeting vector (PG00198_Z_2_G10, project CSD77496) generated by the trans-NIH Knock-Out Project and obtained from the KOMP Repository.

**Statistics**

Statistics were performed using GraphPad Prism software. All datasets were tested for normal distribution with the D’Agostino-Pearson normality test. If a dataset failed this test, a non-parametric test was chosen to compare the significance of means between groups (Mann-Whitney test for two samples and Kruskal-Wallis test for more than two samples). Critical comparative datasets (epothilone treatment, spastin deletion, and shortening of retraction bulbs) were partially re-analyzed in a blinded fashion to exclude observer bias. p values < 0.05 were considered to be significant and indicated by ‘‘*’’; p values < 0.01 were indicated by ‘‘**’’ and <0.001 by ‘‘***’’. Data in text and graphs are given as mean ± SEM, and the chosen statistical test is indicated, along with the corresponding p value, if applicable.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.09.049.

**AUTHOR CONTRIBUTIONS**

M.S.B., T.K., and T.M. conceived of the project and designed experiments. T.M. and M.K. supervised the project. M.S.B., T.K., M.W., N.A.M., and M.S.R. performed imaging experiments. E.W., M.F., M.S.B., and D.L.B. contributed electron microscopy. L.R., T.J.H., and M.K. designed, generated, and characterized the spastin knockout mouse lines. A.A. and S.E. generated AAV vectors. M.S.B., T.K., L.R., T.J.H., D.L.B., N.A.M., M.K., and T.M. designed figures. M.S.B., T.K., and T.M. wrote the paper with input from all authors.

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**REFERENCES**

Alletta, J.M., and Goldberg, D.J. (1982). Rapid and precise down regulation of fast axonal transport of transmitter in an identified neuron. Science 218, 913–916.
Baas, P.W., Rao, A.N., Matamoros, A.J., and Leo, L. (2016). Stability properties of neuronal microtubules. Cytoskeleton 73, 442–460.

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

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., and Schwab, M.E. (2004). The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 7, 269–277.

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

Bishop, D.L., Misgeld, T., Walsh, M.K., Gan, W.B., and Lichtman, J.W. (2004). Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. Science 311, 928–931.

Janke, C., and Bilinskis, J.C. (2011). Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat. Rev. Mol. Cell Biol. 12, 773–786.

Janke, C., and Kneussel, M. (2010). Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton. Trends Neurosci. 33, 362–372.

Kanamori, T., Kanai, M.I., Dairyo, Y., Yasunaga, K., Morikawa, R.K., and Emoto, K. (2013). Compartmentalized calcium transients trigger dendrite pruning in Drosophila sensory neurons. Science 340, 1475–1478.

Kano, M., and Hashimoto, K. (2009). Synapse elimination in the central nervous system. Curr. Opin. Neurobiol. 19, 154–161.

Kasher, P.R., De Vos, K.J., Wharton, S.B., Manser, C., Benett, E.J., Bingley, M., Wood, J.D., Miler, R., McDermott, C.J., Miller, C.C., et al. (2009). Direct evidence for axonal transport defects in a novel mouse model of mutant spastin-induced hereditary spastic paraplegia (HSP) and human HSP patients. J. Neurochem. 110, 34–44.

Kasthuri, N., and Lichtman, J.W. (2003). The role of neuronal identity in synaptogenesis. Nature 424, 426–430.

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.

Kleee, T., Markinkaev, P., Williams, P.R., Stern, S., Weigand, E.E., Engerer, P., Naumann, R., Hartmann, J., Karl, R.M., Bradke, F., et al. (2014). An assay to image neuronal microtubule dynamics in mice. Nat. Commun. 5, 4827.

Kurup, N., Yan, D., Goncharov, A., and Jin, Y. (2015). Dynamic microtubules drive circuit rewiring in the absence of neurite remodeling. Curr. Biol. 25, 1594–1605.

Lacroix, B., van Dijk, J., Gold, N.D., Guizetti, J., Aldrian-Herrada, G., Rogowski, K., Gerlich, D.W., and Janke, C. (2010). Tubulin polyglutamylation stimulates spastin-mediated microtubule severing. J. Cell Biol. 189, 945–954.

Lee, H.H., Jan, L.Y., and Jan, Y.N. (2009). Drosophila Ikk-related kinase Ik2 and Katin p60-like 1 regulate dendrite pruning of sensory neuron during metamorphosis. Proc. Natl. Acad. Sci. USA 106, 6363–6368.

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

Liu, Z., Chen, Y., Wang, D., Wang, S., and Zhang, Y.Q. (2010). Distinct presynaptic and postsynaptic dismantling processes of Drosophila neuromuscular junctions during metamorphosis. J. Neurosci. 30, 11624–11634.

Losonczy, A., Makara, J.K., and Magee, J.C. (2008). Compartmentalized dendritic plasticity and input feature storage in neurons. Nature 452, 436–441.

Luo, L., and O’Leary, D.D. (2005). Axon retraction and degeneration in development and disease. Annu. Rev. Neurosci. 28, 127–156.

Ma, D.L., Chia, S.C., Tang, Y.C., Chang, M.L., Probst, A., Burgunder, J.M., and Tang, F.R. (2006). Spastin in the human and mouse central nervous system with special reference to its expression in the hippocampus of mouse pilocarpine model of status epilepticus and temporal lobe epilepsy. Neurochem. Int. 49, 651–664.

Madsisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zarwala, H.A., Gu, H., Ng, L.L., Palmerit, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. 13, 133–140.

Maar-Nof, M., Homma, N., Raanan, C., Nof, A., Hirokawa, N., and Yaron, A. (2013). Axonal pruning is actively regulated by the microtubule-destabilizing protein kinesin superfamily protein 2A. Cell Rep. 3, 971–977.

Massaro, C.M., Pielage, J., and Davis, G.W. (2009). Molecular mechanisms that enhance synapse stability despite persistent disruption of the spectrin/ankyrin/microtubule cytoskeleton. J. Cell Biol. 187, 101–117.
Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Knochlin, S., et al. (2002). In vivo destablization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J. 21, 6820–6831.

Misgeld, T., Kerschensteiner, M., Bareyre, F.M., Burgess, R.W., and Lichtman, J.W. (2007). Imaging axonal transport of mitochondria in vivo. Nat. Methods 4, 559–561.

Morris, R.L., and Hohenbeck, P.J. (1993). The regulation of bidirectional mitochondrial transport is coordinated with axonal outgrowth. J. Cell Sci. 104, 917–927.

Niwa, S., Takahashi, H., and Hirokawa, N. (2013). α-Tubulin mutations that cause severe neuropathies disrupt axonal transport. EMBO J. 32, 1352–1364.

Perez, F., Diamantopoulos, G.S., Staider, R., and Kreis, T.E. (1999). CLIP-170 highlights growing microtubule ends in vivo. Cell 96, 517–527.

Raineteau, O., and Schwab, M.E. (2001). Plasticity of motor systems after incomplete spinal cord injury. Nat. Rev. Neurosci. 2, 263–273.

Riccomagno, M.M., and Kolodkin, A.L. (2015). Sculpting neural circuits by axon and dendrite pruning. Annu. Rev. Cell Dev. Biol. 31, 779–805.

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.

Rossi, J., Balthasar, O., Olson, D., Scott, M., Berglund, E., Lee, C.E., Choi, M.J., Lauzon, D., Lowell, B.B., and Elmqist, J.K. (2011). Melanocortin-4 receptor expressed by cholinergic neurons regulate energy balance and glucose homeostasis. Cell Metab. 13, 195–204.

Röyttä, M., and Raine, C.S. (1986). Taxol-induced neuropathy: chronic effects of local injection. J. Neurocytol. 15, 483–496.

Ruschej, J., Hellal, F., Flynn, K.C., Dupraz, S., Elliott, D.A., Tedeschi, A., Bates, M., Slivinski, C., Brock, G., Dobrindt, K., et al. (2015). Axonal regeneration. Systemic administration of epothilone B promotes axon regeneration after spinal cord injury. Science 348, 347–352.

Schuldiner, O., and Yaron, A. (2015). Mechanisms of developmental neurite pruning. Cell. Mol. Life Sci. 72, 101–119.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A cre-transgenic mouse strain for the ubiquitous deletion of loxp-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23, 5080–5081.

Sekar, A., Bialas, A.R., de Rivera, H., Davis, A., Hammond, T.R., Kamitaki, N., Tooley, K., Presumey, J., Baum, M., Van Doren, V., et al.; Schizophrenia Working Group of the Psychiatric Genomics Consortium (2016). Schizophrenia risk from complex variation of complement component 4. Nature 530, 177–183.

Sengottuvel, V., Leibinger, M., Pfreimer, M., Andreadaki, A., and Fischer, D. (2011). Taxol facilitates axon regeneration in the mature CNS. J. Neurosci. 31, 2688–2699.

Solowska, J.M., and Baas, P.W. (2015). Hereditary spastic paraplegia SPG4: what is known and not known about the disease. Brain 138, 2471–2484.

Song, J.W., Misgeld, T., Kang, H., Knecht, S., Lu, J., Cao, Y., Cotman, S.L., Bishop, D.L., and Lichtman, J.W. (2008). Lysosomal activity associated with naturally occurring synapse elimination. J. Neurosci. 28, 9893–9901.

Sorbara, C.D., Wagner, N.E., Ladwig, A., Nikic, I., Merkler, D., Kleele, T., Marinkovic, P., Naumann, R., Godinho, L., Bareyre, F.M., et al. (2014). Pervasive axonal transport deficits in multiple sclerosis models. Neuron 84, 1183–1190.

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.

Stepanova, T., Stemmer, J., Hoogenraad, C.C., Lansbergen, G., Dortland, B., De Zeeuw, C.I., Grosveld, F., van Cappellen, G., Akmanova, A., and Galjart, N. (2003). Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). J. Neurosci. 23, 2655–2664.

Tapia, J.C., Wylie, J.D., Kasthuri, N., Hayworth, K.J., Schalek, R., Berger, D.R., Guatimosim, C., Seung, H.S., and Lichtman, J.W. (2012). Pervasive synaptic branch removal in the mammalian neuromuscular system at birth. Neuron 74, 816–829.

Tarrade, A., Fassier, C., Courageot, S., Charvin, D., Vitte, J., Peris, L., Thorel, A., Mouisel, E., Fonknechten, N., Roblot, N., et al. (2006). A mutation of spastin is responsible for swellings and impairment of transport in a region of axon characterized by changes in microtubule composition. Hum. Mol. Genet. 15, 3544–3558.

Testa, G., Schaff, J., van der Hoeven, F., Glaser, S., Anastassiadis, K., Zhang, Y., Hermann, T., Stremmel, W., and Stewart, A.F. (2004). A reliable lacZ expression reporter cassette for multipurpose, knockout-first alleles. Genesis 38, 151–158.

Trotta, N., Orso, G., Rossetto, M.G., Daga, A., and Broadie, K. (2004). The hereditary spastic paraplegia gene, spastin, regulates microtubule stability to modulate synaptic structure and function. Curr. Biol. 14, 1135–1147.

Turney, S.G., and Lichtman, J.W. (2012). Reversing the outcome of synapse elimination at developing neuromuscular junctions in vivo: evidence for synaptic competition and its mechanism. PLoS Biol. 10, e1001352.

Valenstein, M.L., and Roll-Mecak, A. (2016). Graded control of microtubule severing by tubulin glutamylation. Cell 164, 911–921.

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

Watts, R.J., Hooper, E.D., and Luo, L. (2003). Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. Neuron 38, 871–885.

Williams, D.W., and Truman, J.W. (2005). Cellular mechanisms of dendrite pruning in Drosophila: insights from in vivo time-lapse of remodeling dendritic Arborizing sensory neurons. Development 132, 3631–3642.

Wood, J.D., Landers, J.A., Bingley, M., McDermott, C.J., Thomas-McArthur, V., Gleadall, I.J., Shaw, P.J., and Cunliffe, V.T. (2006). The microtubule-severing protein Spastin is essential for axon outgrowth in the zebrafish embryo. Hum. Mol. Genet. 15, 2763–2771.

Yang, G., Lai, C.S., Cichon, J., Ma, L., Li, W., and Gan, W.B. (2014). Sleep promotes branch-specific formation of dendritic spines after learning. Science 344, 1173–1178.

Yaron, A., and Schuldiner, O. (2016). Common and divergent mechanisms in developmental neuronal remodeling and dying back neurodegeneration. Curr. Biol. 26, R628–R639.

Yu, W., Qiang, L., Solowska, J.M., Karabay, A., Korulu, S., and Baas, P.W. (2008). The microtubule-severing proteins spastin and katanin participate differently in the formation of axonal branches. Mol. Biol. Cell 19, 1485–1498.

Zempel, H., Luedtke, J., Kumar, Y., Biernat, J., Dawson, H., Mandelkow, E., and Mandelkow, E.M. (2013). Amyloid-β oligomers induce synaptic damage via Tau-dependent microtubule severing by TTL6 and spastin. EMBO J. 32, 2920–2937.

Zhang, B., Carroll, J.Q., Trojanowski, J.Q., Yao, Y., Iba, M., Potuzak, J.S., Hogan, A.M., Xie, S.X., Ballatore, C., Smith, A.B., 3rd, et al. (2012). The microtubule-stabilizing agent, epothilone D, reduces axonal dysfunction, neurotoxicity, cognitive deficits, and Alzheimer-like pathology in an intervention trial with aged tau transgenic mice. J. Neurosci. 32, 3601–3611.
Supplemental Information

Branch-Specific Microtubule Destabilization Mediates Axon Branch Loss during Neuromuscular Synapse Elimination

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SUPPLEMENTAL FIGURES

Figure S1, related to Figure 1 | Retreating axons branches show reduced anterograde and retrograde transport of mitochondria and peroxisomes. (A) P8 NMJ in a Thy1-mitoCFP-K (mitochondria, cyan) x Thy1-YFP-16 (axoplasm, white) explant with a nearby retraction bulb (indicated by arrowheads; α-bungarotoxin, orange). (B) Anterograde (dark blue) and retrograde (light blue) mitochondrial transport in pre-terminal axons vs. synaptic territory in Thy1-mitoCFP-K x Thy1-YFP-16 mice (n ≥ 24 axons, ≥ 20 mice per group). (C) P9 NMJ in a Thy1-PeroXFP (peroxisomes, yellow) x Thy1-CFP-5 (axoplasm, white) explant with a nearby retraction bulb (indicated by arrowheads; α-bungarotoxin, orange). (D) Anterograde (orange) and retrograde (yellow) transport of peroxisomes in retraction bulbs (‘rebu’) and singly innervated NMJs (‘sin’) of Thy1-PeroXFP x Thy1-XFP mice (n ≥ 16, ≥ 15 mice per group). Scale bar, 5µm in A, also applies to C. Data are mean ± SEM. Mann-Whitney-test was used to determine significance: *** P < 0.001; ** P < 0.01.
Figure S2. related to Figure 1 | Immunohistochemical confirmation of tubulin loss. Various antibodies confirm loss of microtubules, but not neurofilaments, in retreating axon branches, including βIII-tubulin antibodies from mouse (A,B; data replotted from Figure 1G) and rabbit (C,D), α-tubulin antibody (E,F), as well as a neurofilament antibody (G,H). (A,C,E,G) show singly innervated Thy1-YFP-16 NMJs with nearby retraction bulbs and a blow-up of the boxed area; tubulin or neurofilament staining (red) is superimposed on axoplasmic YFP labeling (white) in the merge panel. (B,D,F,H) Quantifications of the respective labeling (as a ratio to YFP) in retreating axon branches (‘rebu’) normalized to axon branches of singly innervated synapses (‘sin’) (B: n ≥ 54 axons from 9 mice per group; D: n ≥ 47, ≥ 3 ; F: n ≥ 28, 4; H: n ≥ 48, 5). (A,C,E,G) The monochrome left hand panels are adjusted with non-linear gamma to enhance visibility of the thin retreating axons; in the right hand panels both channels are linearly adjusted. Scale bars, 5µm throughout. Data are mean ± SEM. Mann-Whitney-test was used in panel B,D,H and an unpaired t-test in panel F was used to determine significance: *** P < 0.001; n.s. P ≥ 0.05.
Figure S3, related to Figure 1 and 2 | Analysis of various time-points across development confirms selective destabilization of the microtubule cytoskeleton in retreating axon branches. Comparison of mitochondrial net transport (A), βIII-tubulin levels (B), EB3 comet density (C) and the EB3/ tubulin ratio (D) in ‘undecided’ (41-60% synaptic territory) axon branches at P7/8, and in axon branches that end in retraction bulbs (‘rebu’) or at singly innervated NMJs (‘sin’) at P9/10 (A: n ≥ 19 axons from ≥ 12 mice; B: n ≥ 20, 5; C: n ≥ 7, 4). Data are mean + SEM. Mann-Whitney-test was used to determine significance: *** P < 0.001; n.s. P ≥ 0.05.
Figure S4, related to Figure 4 and 7 | Epothilone B treatment and genetic ablation of spastin does not cause a developmental delay in the neuromuscular system of postnatal mice. (A-D) Muscle and NMJs of Thy1-YFP-16 mice injected with epothilone B (15µl of 0.5µg/µl) on P4 and examined at P13 do not differ from PEG-injected control. (A) Muscle fiber diameter (n ≥ 148 fibers, 5 mice per group) based on phalloidin staining. (B,C) Plaque-to-pretzel transition staged by appearance of α-bungarotoxin-labeled postsynaptic acetylcholine receptors as 'plaque', 'holes' or 'broken' as quantified in B (n ≥ 217 NMJs, n = 5 mice per group) and illustrated in C (axons, white; α-bungarotoxin, orange). (D) NMJ area following epothilone B treatment (n ≥ 250 NMJs, 5 mice per group). (E-H) Constitutive spastin KO x Thy1-YFP-16 mice are not delayed in neuromuscular development at P11 compared to wild-type litter mates. (E) Muscle fiber diameter (n ≥ 219 muscle fibers, 3 mice per genotype) based on phalloidin staining. (F,G) Plaque-to-pretzel transition quantified in F (n ≥ 150 NMJs, n ≥ 3 mice per genotype) and illustrated in G, as above. (H) NMJ area (n ≥ 150 NMJs, ≥ 3 mice per genotype). Scale bars, 5µm in C, also applies to G; Data are mean ± SEM. Mann-Whitney-test was used to determine significance: * P < 0.05; n.s. P ≥ 0.05.
Figure S5. related to Figure 4 | Epothilone B treatment differentially affects microtubules in terminal axon branches and partially restores organelle transport. (A-C) βIII-tubulin intensities (A), EB3 comet density (B) and ratio of these values (C) in retraction bulbs (‘rebu’) and singly innervated NMJs (‘sin’) is differentially affected by different doses of epothilone B (15μl of indicated concentration; n ≥ 20 axons, ≥ 2 mice per dose for tubulin and n ≥ 13 axons, ≥ 3 mice per dose for EB3 comet density). (D,E) Mitochondrial transport in the anterograde (D) and retrograde (E) direction in axons of the intercostal nerve (ICN; n ≥ 51 axons, ≥ 5 mice), as well as retraction bulbs (‘rebu’; n ≥ 20 axon branches, ≥ 18 mice) or singly-innervating terminal branches (‘sin’; n ≥ 51 axons, ≥ 22 mice). Data are mean + SEM. Kruskal-Wallis-test was used to determine significance in A,B and Mann-Whitney test in D,E: *** P < 0.001; ** P < 0.01; * P < 0.05; n.s. P ≥ 0.05.
Figure S6, related to Figure 6 and 7 | Genetic ablation of spastin does not cause a sprouting phenotype at birth. (A) Number of axon inputs per NMJ in neonatal explants. (B-D) z-projections of tubulin-stained intercostal nerves (ICN) at P0 (B) were used to measure the cross-sectional area (C) and diameter (D) of the nerve. (E-G) α-bungarotoxin-stained muscles (E) were used to measure the width of the endplate band (F) and the NMJ density (G). Scale bars, 10 µm in B, and 100 µm in E; Data are mean ± SEM. Mann-Whitney-test was used to determine significance: n.s. P ≥ 0.05.
SUPPLEMENTAL VIDEO LEGENDS

Movie S1 related to Figure 2 | Time-lapse showing microtubule dynamics in a nerve-muscle explant of a Thy1-EB3-YFP mouse. A singly innervated NMJ with a retraction bulb close-by on the left and a doubly innervated NMJ on the right. Territories of competing inputs were quantified using photo-bleaching (pseudo-colored in magenta and white in still frames at end of movie).

Movie S2 related to Figure 3 | 3D-reconstruction of an NMJ based on serial electron micrographs. A confocal projection following the sequential photo-bleaching procedure shows an axon that occupied 15% (magenta) vs. one that occupied 85% of synaptic territory (white). A surface rendering of a complete scanning transmission electron microscopy reconstruction of the NMJ is overlaid onto the confocal projection. The EM surface rendering is rotated and membrane opacity adjusted to reveal the mitochondria (blue) and microtubules (red) for each axon. Microtubules were rendered only for the axon shaft where all measurements of microtubule dynamics and content were done. Scale cube, 1μm.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

The genotypes and labeling patterns of the animals used in this study are described in the main manuscript.

Generation of spastin knock-out mice

The targeting vector (PG00198_Z_2_G10, project CSD77496) used to generate spastin ‘knockout first’ animals was generated by the trans-NIH Knock-Out Project and obtained from the KOMP repository (www.komp.org). Gene targeting in JM8A3 ES cells, obtained from the European Conditional Mouse Mutagenesis consortium (EUCOMM; Pettitt et al., 2009; Skarnes et al., 2011), was performed according to the protocol provided by the International Knockout Mouse Consortium (IKMC). Positive clones were identified by Southern blot analysis with the 5’-probe on EcoRV digested DNA and by long-range PCR for the 3’-region. Chimeric males were obtained by injection of the ES cells into C57BL6/J 8-cell stage embryos and were mated with C57BL6/J WT female mice to establish germline transmitted founders. Spast<sup>Ytm1at/KOMP</sup>Wtsi offspring were crossed to produce homozygous offspring. Heterozygous ‘knockout-first’ offspring with a minimum of N3 on a C57BL6/J background were inter-crossed to obtain wild-type (WT) and homozygous KO animals. To remove the FRT-flanked neomycin selection cassette these mice were mated with FLPe-Deleter (ACTB-FLPe) mice (Jackson Laboratory #005703; Rodriguez et al., 2000) to obtain the floxed allele used in this study. Subsequent mating with CMV-Cre mice (Jackson Laboratory #006054; Schwenk et al., 1995) employed Cre/loxP recombination for genome-wide inactivation of SPAST.

Definition of the synaptic territory by sequential photo-bleaching

To define the synaptic territory that individual axon branches occupy at a doubly innervated NMJ, we applied a previously published bleaching technique (Brill et al., 2011; Turney and Lichtman, 2012); a video protocol of a closely related procedure can be found in Brill et al., 2013; it is also schematically explained in Figure 1B. In brief, we used Thy1-YFP-<i>J</i> or mice or Thy1-CFP-<i>J</i> in which all motor axons are cytoplasmically labeled, crossed to Thy1-Mito-<i>K</i> or x Thy1-EB3-YFP-J045 mice, respectively, as needed. At a doubly innervated NMJ, the two inputs were bleached sequentially by placing the ‘parked’ laser (515 nm or 440nm, respectively) of a confocal microscope (Olympus FV1000; ×100/1.0 N.A. water-immersion objective) on the preterminal axons. Diffusion allowed the entire presynaptic terminal of the bleached branch to lose fluorescence within 5 - 20s, after which it recovered by replenishment from the cytoplasmic pool. We acquired confocal image stacks after every bleach step and super-imposed the resulting projections using pseudo-colors in Adobe Photoshop. Using this approach, the sum of the synaptic territory of the two inputs regularly exceeded 100% by a small amount (on average by 2.6%) due to overlap, as shown previously (Kashturi and Lichtman, 2003). The total synaptic territory was outlined on the pre-bleach image, with small openings between the preterminal axon branches being disregarded, unless these openings corresponded with a void of bungarotoxin staining in fixed tissue; this is justified on the basis that such openings are typically very transient unless they are reflected on the postsynaptic side (Turney and Lichtman, 2012; Walsh and Lichtman, 2003). Correlated confocal images of fixed synapses were taken following measurements in live nerve-muscle explants.

Imaging of axonal transport, EB3 comet densities and axon shortening in nerve-muscle explants

Triangularis sterni explants were isolated from postnatal day (P) 7-13 mice (both sexes) as previously described (Kerschensteiner et al., 2008). In brief, the skin and pectoral muscles overlying the thorax were dissected. The diaphragm was cut and the anterior thoracic wall was isolated by cutting the ribs close to the vertebral column. The explanted thorax – with the triangularis sterni muscle at the inner wall and its innervating intercostal nerves – was transferred into a dish with oxygenated (95% O<sub>2</sub> 5% CO<sub>2</sub>) Ringer’s solution (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 20 glucose). Thymus, pleura and muscle remnants were removed. The thorax (nerve-muscle explant) was pinned onto a Sylgard-coated 3.5 cm dish using minuten pins, super-fused with warmed oxygenated Ringer’s solution and heated during imaging (33-35°C). Mitochondrial transport and dynamic microtubules in the nerve-muscle explant were assessed using an Olympus BX51WI epifluorescence microscope equipped with a ×20/0.5 N.A. and ×100/1.0 N.A. water-immersion dipping cone objective, an automated filter wheel (Sutter) and a cooled CCD camera (Retiga EXi, Qimaging), controlled by µManager (open
source microscopy software, www.micro-manager.org). Neutral density and infrared-blocking filters in the light path were used to prevent photo-toxicity and photo-bleaching. To follow EB3 ‘comets’, 200 images per movie were acquired at 0.5Hz using an exposure time of 500ms. For axonal transport measurement 180-300 images per movie were acquired at 0.5-1Hz (exposure time of 400-900ms depending on the sample). At least six movies (for mitochondria) and eight movies (for peroxisomes) were taken per experiment. Only one synapse was assessed per nerve-muscle explant to keep the explant time within previously established limits of physiological transport rates (Kerschensteiner et al., 2008).

To determine the speed with which axon branches shortened, mice of the indicated genotypes crossed to Thyl-YFP-16 or Thyl-CFP-5 (pan-motor axonal labelling) were used. Two image stacks of retraction bulbs spanning a time window of 2.5-4.5 h were taken on a slow-scanning confocal laser scanning microscope (Olympus FV1000) equipped with a x100/1.0 N.A. water-immersion objective. Maximum intensity projections were generated using the open source software ImageJ/Fiji (http://fiji.sc) to determine the distance by which the axon branch shortened.

**Neonatal virus labeling**

For postnatal deletion of spastin, AAV injections were performed on P1 or 2 in spastin<sup>fl/fl</sup> or control animals crossed to CAG-tdTomato reporter mice (Jackson Laboratory #007914, Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J) to monitor recombination. Pups were briefly anesthetized with isofluorane and injected with 3 µl of AA9-CMV-iCre (codon-improved cre recombinate; titer: 8 x 10<sup>9</sup>/µl) into the 3<sup>rd</sup> ventricle using a nanoliter injector attached to a fine glass pipette. The injection was guided by ultrasound (Visualsonics, Vevo® 2100). Mice were then placed on a warming pad and returned to the mother after recovery. For analysis, putatively spastin-deleted axon branches (i.e. tdTomato-expressing axons in a spastin<sup>fl/fl</sup> background) were compared with axon branches that either did not express tdTomato, or were in a genetic back-ground not carrying the floxed allele.

**Epothilone B injection**

For microtubule dynamics and βIII-tubulin measurements, Thyl-YFP-16 and Thyl-EB3-YFP-J045 mice were injected subcutaneously using a 30G insulin needle with 15µl vehicle (PEG 300, Sigma) or 15µl of epothilone B (0.1, 0.25 or 0.5µg/µl; Selleckchem; dissolved in vehicle). Poly-innervation was quantified in Thyl-YFP-16 on P7, 9, 11, 13 and 21 (injection on P4). For analysis of EB3 ‘comet’ densities and βIII-tubulin levels, injections were done on P5, as EB3 labeling proved too dim for analysis before P6. EB3 densities and βIII-tubulin levels were analyzed on P6 to assay the maximum effect of this treatment. The experimenter was blind to the treatment during scoring, imaging and analysis.

**Immunohistochemistry and confocal microscopy**

For immunohistochemistry the whole thorax was fixed in 4% paraformaldehyde (PFA) or formaldehyde in 0.1M phosphate buffered saline (PBS) or 0.1M phosphate buffer (PB). See details for each antibody below. The triangularis muscle was dissected (Brill et al., 2013; Kerschensteiner et al., 2008) and incubated overnight (4°C) in the following primary antibodies diluted in blocking solution: anti-βIII-tubulin conjugated to Alexa555 (mouse monoclonal, 1:200; BD Pharmingen, #560339; fixation 4% PFA in 0.1M PBS for 1hr; blocking solution 10% GS, 1% BSA, 0.3% Triton X-100 in 0.1M PBS); anti-βIII-tubulin (rabbit polyclonal, 1:1000; Covance, PRB-435P; fixation 4% PFA in 0.1M PB for 1-2hrs; blocking solution 1% BSA, 10% goat serum, 0.5% Triton X-100 in 0.1M PB); anti-α-tubulin (rabbit polyclonal, 1:500; Synaptic Systems, #302203; fixation 4% PFA in 0.1M PB for 1hr; blocking solution 5% BSA, 0.5% Triton X-100 in 0.1M PB); anti-tyrosinated α-tubulin (rat monoclonal, 1:1000; AbD Serotec, YL1/2, #MCA77G; fixation 4% formaldehyde in 0.1M PB for 1hr; blocking solution 5% BSA, 0.5% Triton X-100 in 0.1M PB); anti-glutamylated α-tubulin (mouse monoclonal, 1:200; Synaptic System, 1D5, #302011; fixation 4% PFA in 0.1M PB for 2hrs; blocking solution 1% BSA, 10% goat serum, 0.5% Triton X-100 in 0.1M PB); anti-acetylated α-tubulin (mouse monoclonal, 1:1000; Sigma, clone 6-11B-1, #T7451; fixation 4% formaldehyde in 0.1M PB for 3hrs; blocking solution 5% BSA, 0.5% Triton X-100 in 0.1M PB); anti-polyglutamate chain (polyE; rabbit polyclonal, 1:400; Adipogen, #AG-25B-0030-C050; fixation 4% formaldehyde in 0.1M PB for 2hrs; blocking solution 1% BSA, 10% goat serum, 0.5% Triton X-100 in 0.1M PB); anti-200KD Neurofilament Heavy (rabbit polyclonal, 1:500; Abcam, #ab8135; fixation 4% PFA in 0.1M PB for 1hr; blocking solution 1% BSA, 10% goat serum, 0.5% Triton X-100 in 0.1M PB). Muscles were washed in 0.01M PBS or 0.1M PB, respectively, incubated for 1hr at room temperature with suitable secondary antibodies coupled to Alexa 647, Alexa 594, Alexa
568 or Alexa 488 (Invitrogen), and washed in 0.01M PBS or 0.1M PB, respectively, depending on the blocking solution. To label postsynaptic nicotinic acetylcholine receptors, muscle fibers or cell bodies, we incubated the samples in Alexa 594- or Alexa 488-conjugated α-bungarotoxin (Invitrogen, B-13423, B-35450, 50µg/ml, 1:50 in PBS), Alexa 647-conjugated phalloidin (Invitrogen, A22287, 200units/ml methanol, 1:50 in PBS), or NeuroTrace 640/660 (Invitrogen, N-21483, 1:500), respectively. Entire triangularis sterni muscles were mounted in Vectashield (Vector Laboratories). Image stacks were recorded using a confocal microscope (Olympus FV1000) equipped with x10/0.40 N.A. air, x20/0.8 N.A., x40/1.35 and x60/1.42 N.A. oil-immersion objectives, and maximum intensity projections were generated using the open source software ImageJ/Fiji (http://fiji.sc).

**Image processing/representation**

For figure representation, different channels of confocal image series were combined in pseudo-color using the ‘screen’ function in Adobe Photoshop. In non-quantitative panels, gamma was adjusted non-linearly to enhance visibility of low-intensity objects. Images were placed on dark monochrome backgrounds where appropriate, with the boundary visible due to a non-clipped noise background in all images.

**Southern blot analysis**

Southern blot analysis was performed using an external probe amplified by PCR from the genomic region adjacent to the 5’ homologous arm of the targeting vector. The following 5’-probe was used: AGCTTTACATTCCAAGAAGATACATTTTAATAGTGTCAT. Genomic DNA was isolated from tail biopsies using a DNA isolation reagent for genomic DNA (Applichem), digested with EcoRV for analysis of the 5’-region. The DNA was separated on a 0.8% agarose gel, blotted onto Hybond-XL (Amersham) by alkaline transfer, and hybridized to the random-primed alpha-32P-labeled probe.

**Long range PCR and genotyping**

Genomic DNA was extracted from tail biopsies using standard protocols and processed for PCR. The following primers were used for the 3’ long-range PCR revealing a 5043 bp product: Sense (primer 4): 5’-TACATTACAGAAGTATGGTCT-3’ (complement to loxP site) and antisense (primer 5): 5’-TCTACATCCCACCTAGTGGAATCATCT-3’ (complement to genomic region). The following primers were used for the genotyping PCR: WT sense (primer 2): 5’-ATTTCAGAAAAACTACTTGCTATTAATTCC-3’; WT and KO antisense (primer 3): 5’-CACATGGTGCTCATATAACCATTATA-3’; KO sense (primer 1): 5’-AAGTCTAGGCAGTCTTTTCTGGCT-3’. The presence of the wild-type and knockout alleles was indicated by 223 and 432 bp products, respectively, which were detected on a 1.5 - 2% agarose gel.

**Analysis of spastin protein expression levels**

Spinal cord of homozygous KO, heterozygous and wild-type spastin knockout littermate mice were dissected as follows. All steps after tissue extraction were performed at 4°C: Spinal cords were removed after animals were killed, washed once in PBS and transferred into lysis buffer containing 1% (v/v) Triton X-100 in PBS. Tissue was homogenized using a hand disperser with a 5mm aggregate and a tip speed of 5m/s for 10s. After incubation for 30min on a rotating wheel, lysates were centrifuged at 1,000 x g for 10min. The resulting supernatant was boiled in SDS sample buffer after adjustment of protein concentrations using a BCA assay (Pierce Biotechnology). Samples were analyzed by Western blotting using the following antibodies: mouse anti-spastin (1:500; Santa Cruz, sc-81624); rabbit anti-actin (1:500; Sigma, A2066); peroxidase-conjugated goat anti-mouse and anti-rabbit (1:10,000; Dianova).

**Electron microscopy**

After sequential photo-bleaching of doubly innervated NMJs using Thy1-YFP-16 mice, a 'map' was recorded and nerve-muscle explants were fixed in 2.5% glutaraldehyde and 2% PFA in 0.1M PBS. After 24hrs post-fixation at 4°C thoraxes were transferred to 0.1M Sorensen’s-phosphate buffer (pH 7.4), the triangularis muscle was excised as described above, and mounted in Sorensen’s-phosphate buffer. The NMJ of interest was identified by the map recorded earlier and DiI crystals were placed close-by (Bishop et al., 2004). For electron microscopy, muscles were
stained with 1% osmium-tetroxide reduced in 1.5% potassium ferrocyanide in 0.1M sodium cacodylate for 15min followed by 1% osmium tetroxide for 45min, washed again in three changes of 0.1M sodium-cacodylate buffer, dehydrated in an ascending ethanol series and two changes in 100% propylene oxide, embedded in Araldite502/Embed812 resin and polymerized at 60°C for 48hrs. Tissue blocks were flat-embedded in BEEM capsules and oriented for correlated light and electron microscopy as previously described (Gan et al., 1999). Serial ultrathin sections (~60nm) were collected onto pioloform-coated slot grids, and counterstained with 2% aqueous uranyl acetate and Reynold’s lead citrate (30min each). Electron micrographs were obtained at 120KV using a transmission electron microscope at a resolution of 1.6nm per pixel (JEOL, JEM-1400 equipped with a Gatan Ultrascan 1000XP camera; Kleele et al., 2014; Sorbana et al., 2014). Serial sections were aligned and analyzed using the Reconstruct software at SynapseWeb (http://synapses.clm.utexas.edu/). Section thickness was measured using the cylindrical diameters method (Fiala and Harris, 2001). Microtubule density was quantified by measuring the sum of all microtubule lengths divided by the respective axon volume. Surface renderings from serial section datasets were generated using 3dMax (Autodesk). Graph in Figure 3 represents eight pre-terminal axons quantified from four NMJs, with one NMJ having three inputs and one NMJ where only one of the two inputs was quantified.

Data analysis

Movies were aligned using the ‘StackReg’ algorithm (available in Fiji). EB3 ‘comets’ were tracked manually using the ‘MTrackJ’ plugin (developed by E.Meijering, Biomedical Imaging Group, Erasmus Medical Center, Rotterdam) available in Fiji. Fluorescent EB3 ‘comets’ that appeared at least in three consecutive frames were included into the analysis. The number of times comets were observed in one region of interest (i.e. an in-focus axon area) was summed and divided by the number of analyzed frames and the observed area of the axon in µm². In the figures, graphs show number of comets per axon area (± SEM of all axons analyzed). Images in Figures 2 C and E represent maximum intensity projection of 20s within a time-lapse sequence.

To determine transport rates, i.e. the number of mitochondria or peroxisomes travelling in the anterograde or retrograde direction, we counted the number of fluorescent organelles, which passed a region in focus of the axon quantified. The net transport is calculated as the rate of mitochondria moving anterograde subtracted by the rate of retrograde moving mitochondria per axon. In the figures, graphs represent mitochondria or peroxisomes per hour (± SEM) of all axons analyzed.

For quantifying the amount of α-/βIII-tubulin and neurofilament in individual axons by immunohistochemistry, the triangularis stern muscle of Thy1-YFP-16 transgenic mice was stained with tubulin antibodies (as described above) and confocal image stacks were analyzed in Fiji. Post-translational tubulin modifications were normalized in a double staining for a modification and βIII-tubulin. For quantification, regions of interest (ROIs) were placed in a single optical section within an axon and mean grey values were averaged for every channel. For βIII-tubulin in Figure 1G and Figure 5I, at least two ROIs were placed in a distinct single optical section of the confocal stack within the axon. Both channels (YFP16 and tubulin) were background-subtracted in every single optical section. The ratio of the average grey values was determined following by a second normalization on a singly innervating axon within the field of the view (to correct for staining variability between samples and analyzed areas). For Figure S2, tubulin and neurofilament stainings were normalized once, either on YFP or βIII-tubulin, respectively. In Figure 5A-H, two or more ROIs were analyzed, but only one background subtraction was made, namely in the ROI that was closest to the muscle surface.

To control for toxic effects of epothilone B or alterations in spastin KO mice in the triangularis sterni muscle, we analyzed the average size and appearance of NMJs in confocal image stacks of fixed muscles (Breckwoldt et al., 2014; Kleele et al., 2014). We took Thy1-YFP-16 x spastin KO mice and compared them to Thy1-YFP-16 x spastin WT litter-mates or analyzed muscles from Thy1-YFP-16 epothilone B or vehicle (PEG) injected animals. Following fluorescently tagged α-bungarotoxin staining to label the postsynaptic acetylcholine receptors, the size of flat NMJs was determined in maximum intensity projections using the ‘Otsu’ threshold algorithm and the ‘wand’ tool available in Fiji. Synapse maturation was monitored by subdividing their appearance into three classes: plaque (no area lacking fluorescence in a roughly circular or elliptical area), holes (one or more areas devoid of fluorescence) and broken (one or more openings continuous with the outside, resulting in C or W shapes). We considered plaques as immature NMJs due to the developmental plaque-to-pretzel transition (Marques et al., 2000). The appearance was scored manually without algorithm. The diameter of the muscle fibers was measured based on phalloidin staining on orthogonal (xz) confocal projections: three diameters in different directions on each fiber were averaged to account
for muscle fibers not being circular. The experimenter was blind to the treatment during scoring, imaging and analysis.

To exclude prenatal effects of spastin deletion on the branching pattern of developing motor units, we used well-established analysis approaches used to study neuromuscular synapse formation (Misgeld et al., 2002; Misgeld et al., 2005). We imaged the endplate band of the triangularis sterni muscle in thorax whole mounts of P0 pups of spastin knock-out and control mice. These preparations were stained with α-bungarotoxin and anti-βIII-tubulin as indicated above. A polygon was drawn to connect the most peripheral NMJs, and the endplate width was calculated by dividing the polygon area by its height orthogonal to the myofiber long axis; at the same time the synapse density was determined per field of view. The number of inputs per synapse at birth was estimated in tubulin stains; while this provides only a lower estimate of the number of inputs due to fasciculation (Tapia et al., 2012), this measure is sensitive to neonatal sprouting effects (Misgeld et al., 2002). In tubulin stainings, also the calibre and cross-sectional area of the second intercostal nerve was measured as a surrogate read-out of axon numbers (Misgeld et al., 2002), using the 'reslice' function of ImageJ to generate xz views from confocal stacks, and the diameter/area was determined by averaging three measurements at different positions along the nerve.

Statistics

The statistical methods are described in the main manuscript.
SUPPLEMENTAL REFERENCES

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.

Breckwoldt, M.O., Pfister, F.M., Bradley, P.M., Marinkovic, P., Williams, P.R., Brill, M.S., Pomer, B., Schmalz, A., St Clair, D.K., Naumann, R., et al. (2014). Multiparametric optical analysis of mitochondrial redox signals during neuronal physiology and pathology in vivo. Nature Medicine 20, 555-560.

Brill, M.S., Lichtman, J.W., Thompson, W., Zuo, Y., and Misgeld, T. (2011). Spatial constraints dictate glial territories at murine neuromuscular junctions. The Journal of Cell Biology 195, 293-305.

Brill, M.S., Marinkovic, P., and Misgeld, T. (2013). Sequential photo-bleaching to delineate single Schwann cells at the neuromuscular junction. Journal of Visualized Experiments e4460.

Gan, W.B., Bishop, D.L., Turney, S.G., and Lichtman, J.W. (1999). Vital imaging and ultrastructural analysis of individual axon terminals labeled by iontophoretic application of lipophilic dye. Journal of Neuroscience Methods 93, 13-20.

Kasthuri, N., and Lichtman, J.W. (2003). The role of neuronal identity in synaptic competition. Nature 424, 426-430.

Kerschensteiner, M., Reuter, M.S., Lichtman, J.W., and Misgeld, T. (2008). Ex vivo imaging of motor axon dynamics in murine triangularis sterni explants. Nature Protocols 3, 1645-1653.

Kleele, T., Marinkovic, P., Williams, P.R., Stern, S., Weigand, E.E., Engerer, P., Naumann, R., Hartmann, J., Karl, R.M., Bradke, F., et al. (2014). An assay to image neuronal microtubule dynamics in mice. Nature Communications 5, 4827.

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., Ng, L. L., Palmiter, R. D., Hawrylycz, M. J., Jones, A. R. et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nature Neuroscience 13, 133-140.

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. The Journal of Neuroscience 20, 3663-3675.

Misgeld, T., Burgess, R. W., Lewis, R. M., Cunningham, J. M., Lichtman, J. W., Sanes, J. R. (2002). Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. Neuron 36, 635-48.

Misgeld, T., Kummer, T. T., Lichtman, J. W., Sanes, J. R. (2005). Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. Proceedings of the National Academy of Sciences of the United States of America 102, 11088-93.

Pettitt, S.J., Liang, Q., Rairdan, X.Y., Moran, J.L., Prosser, H.M., Beier, D.R., Lloyd, K.C., Bradley, A., and Skarnes, W.C. (2009). Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nature Methods 6, 493-495.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nature Genetics 25, 139-140.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Research 23, 5080-5081.
Skarnes, W.C., Rosen, B., West, A.P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A.O., Thomas, M., Harrow, J., Cox, T., et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474, 337-342.

Sorbara, C.D., Wagner, N.E., Ladwig, A., Nikic, I., Merkler, D., Kleele, T., Marinkovic, P., Naumann, R., Godinho, L., Bareyre, F.M., et al. (2014). Pervasive axonal transport deficits in multiple sclerosis models. Neuron 84, 1183-1190.

Tapia, J. C., Wylie, J. D., Kasthuri, N., Hayworth, K. J., Schalek, R., Berger, D. R., Guatimosim, C., Seung, H. S., Lichtman, J.W. (2012). Pervasive synaptic branch removal in the mammalian neuromuscular system at birth. Neuron 74, 816-829.

Turney, S.G., and Lichtman, J.W. (2012). Reversing the outcome of synapse elimination at developing neuromuscular junctions in vivo: evidence for synaptic competition and its mechanism. PLoS Biology 10, e1001352.

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.