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### Citation for published version:

Wang, M, Kleele, T, Xiao, Y, Plucinska, G, Avramopoulos, P, Engelhardt, S, Schwab, MH, Kneussel, M, Czopka, T, Sherman, D, Brophy, PJ, Misgeld, T & Brill, MS 2021, 'Completion of neuronal remodeling prompts myelination along developing motor axon branches', *Journal of Cell Biology*.  
<https://doi.org/10.1083/jcb.201911114>

### Digital Object Identifier (DOI):

[10.1083/jcb.201911114](https://doi.org/10.1083/jcb.201911114)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Journal of Cell Biology

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# Completion of neuronal remodeling prompts myelination along developing motor axon branches

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*Running title:* Myelination during axonal remodeling

*Keywords:* Myelin, nodes of Ranvier, plasticity, synapse elimination, neuromuscular junction, axon, Schwann cells, Caspr, Neuregulin

*Characters:* 27,365 (characters not including spaces, without methods and references)

*Figures:* 7 (+ 4 Suppl. Figures)

## 1 **SUMMARY**

2 Postnatal motor neurons undergo extensive competitive remodeling and synchronously myelinate.  
3 Wang et al. now reveal that axon remodeling and myelination intersect: While myelination does not  
4 predetermine competition outcome, completing remodeling allows myelination to accelerate. This  
5 involves cytoskeletal maturation, which enables increased delivery of pro-myelinating signals.

6

## 7 **ABSTRACT**

8 Neuronal remodeling and myelination are two fundamental processes during neurodevelopment.  
9 How they influence each other remains largely unknown, even though their coordinated execution is  
10 critical for circuit function and often disrupted in neuropsychiatric disorders. It is unclear, whether  
11 myelination stabilizes axon branches during remodeling or whether ongoing remodeling delays  
12 myelination. By modulating synaptic transmission, cytoskeletal dynamics and axonal transport in mouse  
13 motor axons, we show that local axon remodeling delays myelination onset and node formation.  
14 Conversely, glial differentiation does not determine outcome of axon remodeling. Delayed myelination is  
15 not due to a limited supply of structural components of the axon-glial unit, but rather triggered by  
16 increased transport of signaling factors that initiate myelination, such as neuregulin. Further, transport of  
17 pro-myelinating signals is regulated via local cytoskeletal maturation related to activity-dependent  
18 competition. Our study reveals an axon branch-specific fine-tuning mechanism that locally coordinates  
19 axon remodeling and myelination.

## 20 **INTRODUCTION**

21 Myelin enables saltatory conduction and provides trophic support to the sheathed axons ([Huxley and](#)  
22 [Stämpeli, 1949](#); [Vabnick and Shrager, 1998](#); [Yin et al., 2006](#); [Simons and Trotter, 2007](#); [Nave, 2010](#)). In

23 addition, recent observations in the central nervous system (CNS) indicate that myelin contributes to fine-  
24 tuning of neural circuits (Fields, 2015; Chang et al., 2016; Kaller et al., 2017). For instance, myelin sheaths  
25 and nodes of Ranvier — ion channel-enriched axon segments interspersed between myelin sheaths —  
26 show activity-dependent plasticity (Huff et al., 2011; Gibson et al., 2014; Mensch et al., 2015; Etxeberria  
27 et al., 2016; Korrell et al., 2019; Bacmeister et al., 2020) that *e.g.* appear to shape ‘patchy’ myelination  
28 patterns in neocortex (Tomassy et al., 2014). While activity-regulated myelination is less studied in the  
29 peripheral nervous system (PNS, Stevens and Fields, 2000; Fields, 2015), in the PNS, the axon-glia unit is  
30 more accessible than in the CNS, and the signaling pathways governing peripheral myelination are better  
31 understood (Taveggia et al., 2010; Pereira et al., 2012; Grigoryan and Birchmeier, 2015) . Thus, PNS  
32 development offers a privileged window into the intersection of axonal remodeling and myelin plasticity.

33 To capitalize on these advantages, we turned to a major site of PNS remodeling, the neuromuscular  
34 junction (NMJ). At mouse NMJs, axonal remodeling follows a predictable course during the first two  
35 postnatal weeks and can be followed at the single axon branch level (Lichtman and Sanes, 2003; Walsh  
36 and Lichtman, 2003). At birth, multiple motor axon branches innervate the same postsynaptic site (Tapia  
37 et al., 2012). Subsequently all but one of these presynaptic inputs are eliminated by a two-step process  
38 that first involves activity-driven competition and then axon branch removal by cytoskeletal degradation  
39 (Buffelli et al., 2003; Brill et al., 2016), until lifelong innervation by a single axon is established (Tapia and  
40 Lichtman, 2012). Already during embryonic development, Schwann cells (SCs)—the glia of the PNS—  
41 surround growing motor axons and accompany them to the target muscle (Jessen and Mirsky, 2005). SCs  
42 initiate myelination perinatally, after SCs have been sorted to sheath individual axon branches (Jessen and  
43 Mirsky, 2005; Monk et al., 2015; Rasband and Peles, 2016). Overall, myelination follows a proximal-to-  
44 distal gradient along motor axons with myelination of terminal branches occurring last and asynchronously  
45 (Hildebrand et al., 1994; Yamamoto et al., 1996). This temporal correlation between axon-glia  
46 differentiation and cessation of developmental axon plasticity is a general feature across the nervous



47 system, and in the CNS can e.g. be observed in visual cortex (Luo and O’Leary, 2005; McGee et al., 2005;  
48 Simons and Trotter, 2007). In the PNS, myelination onset is determined by the level of Neuregulin-1 (Nrg1)  
49 type III on the axonal surface. Nrg1 binds to glial ErbB2/3 receptors on SCs, leading to phosphorylation of  
50 down-stream effectors, such as ERK1/2 and AKT (Garratt et al., 2000; Michailov et al., 2004; Taveggia et  
51 al., 2005; Iwakura and Nawa, 2013; Basak et al., 2015). While Nrg1 signaling is known to affect synapse  
52 development, the underlying signaling takes place at the NMJ itself, involving ‘terminal’ non-myelinating  
53 SCs, rather than myelinating SCs along the axon (Loeb, 2003; Hayworth et al., 2006; Lee et al., 2016). Thus,  
54 whether the timing of branch-specific myelination also depends on local availability of Nrg1, and whether  
55 Nrg1 signaling is locally regulated to coordinate axon remodeling and myelination remains to be elucidated.  
56 Hence, using the NMJ as a model, we asked: How are axonal competition and axon-glia differentiation  
57 coordinated at the single-branch level, and what is the signaling mechanism involved?

## 58 RESULTS

### 59 Axon-glia differentiation is delayed on branches engaged in remodeling

60 To study the intersection of axon remodeling and myelination, we took advantage of a thoracic nerve-  
61 muscle explant, including the *triangularis sterni* muscle, which is uniquely suited to study the cell biological  
62 dynamics of single axon branches (Kerschensteiner et al., 2008; Brill et al., 2013; Fig. 1 A). During the  
63 second postnatal week, most NMJs transition from double to single innervation (abbreviated in the figures  
64 as ‘din’ and ‘sin’, respectively), while myelin and nodes of Ranvier appear on terminal branches (Fig. 1 B).  
65 On three postnatal days (P7, 9 and 11), we quantified the number of NMJs still engaged in synaptic  
66 competition using *triangularis sterni* muscles derived from *Thy1-XFP* mice, where motor axons are  
67 fluorescently labeled (Feng et al., 2000; Fig. 1, C and D). In parallel, we determined the state of axon-glia  
68 differentiation on singly innervating terminal branches based on the presence of immunostained Caspr1  
69 (contactin-associated protein 1; Rasband and Peles, 2016) accumulations along a given terminal branch.

70 We did not distinguish further between fully formed nodes (paranodes on both sides) and partially formed  
71 heminodes, resulting in a binary score (Caspr+ vs. Caspr- terminal branches; [Fig. 1, C and D](#)). We observed  
72 a concomitant resolution of synaptic competition (*i.e.* decreasing percentage of doubly innervated  
73 synapses) and increasing paranodal formation on ‘winner’ branches ([Fig. 1 D](#)). Finally at P13, all NMJs  
74 established single innervation ( $100 \pm 0\%$ ,  $n = 3$  mice, 100 NMJs per animal) and all terminal branches  
75 started to form paranodes ( $100 \pm 0\%$ ,  $n = 3$  mice, 30 NMJs per animal). Next, we immunostained for myelin  
76 and other nodal components in *Thy1-XFP* mice at P9. In parallel to Caspr, nodal (voltage-gated sodium  
77 channel, Nav), juxta-paranodal (contactin-2, CNTN2), and internodal (myelin protein zero, MPZ) markers  
78 emerged on terminal branches ([Fig. 1, E and F](#); [Doyle and Colman, 1993](#)). Thus, as myelin and nodal  
79 compartments co-assemble rapidly ([Girault and Peles, 2002](#); [Schafer et al., 2006](#)), we used Caspr  
80 immunostaining as a surrogate for overall axon-glia differentiation. Notably, when we focused on the  
81 branches still engaged in competition, we found significantly fewer branches immuno-positive for  
82 emerging nodal structures, resulting in an overall delay of axon-glia differentiation of roughly two days  
83 ( $\sim 33\%$  of the full 6-day myelination period) compared to their winner siblings. This finding was consistent  
84 across all markers tested ([Fig. 1 F](#)). Thus, ongoing competition, and hence sustained plasticity, of terminal  
85 axon branches coincides with a transient stall of myelination and node formation. We considered two  
86 explanations for this delay: (1) Slower assembly of structural components of the axon-glia unit, or (2)  
87 reduced pro-myelinating signals. To disambiguate these scenarios, we analyzed the dynamics of node  
88 formation during axonal remodeling.

### 89 **Axon remodeling delays initiation, not progress of axon-glia differentiation**

90 To measure the progress of axon remodeling, as well as the onset of node formation on individual  
91 motor axon branches, we characterized transgenic mice expressing GFP-tagged Caspr (Caspr-GFP; [Fig. S1](#);  
92 [Brivio et al., 2017](#)) and generated mice expressing the  $\beta 1$  subunit of the voltage-gated sodium channel

93 tagged with GFP ( $\beta$ 1-Nav-GFP; Fig. S1), both under control of the *Thy1* promoter. In both lines, progress of  
94 synapse elimination and onset of node formation were unchanged compared to wild-type littermates at  
95 P9 (Fig. S1 legend). We assessed the paranodal/nodal protein dynamics by fluorescence recovery after  
96 photobleaching (FRAP, see Methods), and related the recovery rate to axonal competition status in  $\beta$ 1-  
97 Nav- or Caspr-GFP crossed to *Thy1*-OFP3 mice (Brill et al., 2011). GFP-positive clusters forming heminodes  
98 were photo-bleached to approximately one third of their original fluorescence intensity ( $33 \pm 3\%$ ,  $n = 135$   
99 nodes in 46 mice) and visualized again three hours later. The recovery was normalized to non-bleached  
100 control nodes in the same field of view to account for imaging-related fluorescence loss (Fig. 2, A and C).  
101 Surprisingly, at P9-11 we found significantly higher recovery rates of  $\beta$ 1-Nav- or Caspr-GFP on competing  
102 doubly innervating branches compared to singly innervating ones (Fig. 2, B and D) — suggesting that once  
103 initiated, node formation progressed swiftly. We also found an age dependent decline (Rios et al., 2000):  
104 Recently established nodal structures recover much faster than mature ones ('sin' P9-11 vs. 'sin' 6wk;  
105 Caspr-GFP  $\sim 4.3$  fold;  $\beta$ 1-Nav-GFP  $\sim 4.4$  fold; Zhang et al., 2012). At the same time, P9-11 nodes in proximal  
106 positions ('stem'; Fig. 2, B and D) resembled distal mature (i.e. 6wk) nodes, consistent with the known  
107 myelination gradient (Hildebrand et al., 1994). Hence, our data favor a mechanism where axonal  
108 competition delays initiation, but not progress of axon-glia maturation. However, myelination and node  
109 formation are still initiated on a subset of competing, doubly innervating axon branches (cf. Fig. 1 F). Thus  
110 we wondered, whether disparate progress of axon-glia maturation influences the competition outcome.

### 111 **Axon-glia maturation does not convey an advantage in synaptic competition**

112 To address the effect of a branch's axon-glia maturation status on competition, we related initiation of  
113 node formation to synaptic territory (i.e. the fraction of an NMJ that a terminal branch innervates).  
114 Synaptic territory is a well-established indicator of probable competition outcome (Gan and Lichtman,  
115 1998; Walsh and Lichtman, 2003; Brill et al., 2016). We determined synaptic territory using the 'Brainbow'

116 approach to individually color motor units (*ChAT-Cre* x *Thy1-Brainbow-1.1*; Fig. 3 A; Livet et al., 2007; Rossi  
117 et al., 2011) and immunostained for Caspr to reveal node formation along terminal branches at P9.  
118 Throughout all stages of axonal competition (1-99% territory), less than ~20% of the branches were Caspr-  
119 positive, and there was no correlation of myelination onset to synaptic territory (Fig. 3 B). However, once  
120 competition was resolved, Caspr was present on ~50% of the singly innervating terminal branches (100%  
121 territory;  $\chi^2$  test, 1-99% vs. 100%,  $p < 0.0001$ ,  $n = 749$  axon branches in 45 mice; Fig. 3 B), suggesting a swift  
122 lift of the brake on axon-glia differentiation once competition was resolved. This lack of correlation  
123 contrasts with other cell biological features of terminal branches, e.g. cytoskeletal stability, organelle  
124 transport or caliber, which are highly correlated to synaptic territory (Keller-Peck et al., 2001; Brill et al.,  
125 2016). Moreover, the measured distribution of node formation patterns on competing axons, e.g. the  
126 fraction of NMJs where node formation had started on the winning (51-99% territory), the losing (1 – 49%  
127 territory), or neither of the competing branches, matched a random binomial distribution (14% ‘din’  
128 myelinated across 1-99% territory,  $n = 520$  ‘din’ NMJs in 35 mice; Fig. 3 C). This, however, does not rule  
129 out caliber as a central driver of node formation onset, as known for other PNS settings, where 1  $\mu\text{m}$   
130 represents a critical threshold for myelination (Voyvodic, 1989; Peters et al., 1991). Therefore we analyzed  
131 the diameter of competing branches with or without emerging nodes (Fig. 3 D), and found no difference  
132 between the groups during all stages of competition. We even found—albeit rarely—partially myelinated  
133 axon branches that lost against a non-myelinated competitor (Fig. 3, F and G). Only in retreating branches  
134 were the pruning axons with emerging nodes significantly thicker than their unmyelinated counterparts  
135 (Fig. 3 D), possibly due to the protective effect of myelin on axonal structures, as axon stretches covered  
136 by MPZ are significantly thicker than MPZ negative stretches along the same retreating branches (Fig. 3 E;  
137 Nave, 2010). Hence, axon-glia differentiation neither decides competition, nor prohibits axon pruning (cf.  
138 McGee et al., 2005). Together, the data suggest a unidirectional relationship, with ongoing axon  
139 remodeling delaying axon-glia maturation, but not the converse. Next, we wanted to test which phase of

140 synapse remodeling impacted axon-glia maturation to narrow down possible underlying signaling  
141 mechanisms.

## 142 **Suspending competition, but not late branch removal, delays axon branch myelination**

143 Synapse remodeling can be divided into several phases (Kano and Hashimoto, 2009; Turney and  
144 Lichtman, 2012), with an early activity-dependent competition phase driven by neurotransmission (Buffelli  
145 et al., 2003), followed by a late execution phase involving cytoskeletal break-down and glial engulfment  
146 (Bishop et al., 2004; Brill et al., 2016). First, we intervened during competition by irreversibly blocking  
147 postsynaptic acetylcholine receptors (AChRs) using unilateral thoracic injection of  $\alpha$ -bungarotoxin (BTX,  
148 Akaaboune et al., 1999; Kummer et al., 2004) of P7 *Thy1*-XFP mice (Fig. 4 A). Paired analysis on contra- vs.  
149 ipsilateral *triangularis sterni* muscles two days later (P9; Fig. 4, B and C) revealed that more multiple  
150 innervation was maintained after BTX injection (Fig. 4 D; Loeb et al., 2002; Buffelli et al., 2003). Notably,  
151 the number of Caspr-positive winner branches ('sin') was significantly reduced (Fig. 4 E), suggesting that  
152 blocking neurotransmission delays the initiation of node formation. At the same time, we neither  
153 measured a change of axonal SC number, nor of internode or terminal branch length after BTX treatment  
154 (Fig. S2, A–D). Under physiological condition, the number of SCs slightly increases as competition resolves  
155 (indicated by reduced SC length; Fig. S2, E and F). We therefore hypothesized that BTX injection maintained  
156 terminal branches in a more juvenile state. Thus, we turned to the microtubular cytoskeleton as an  
157 important indicator for axonal maturation, since microtubular mass increases as the branch gains synaptic  
158 territory (Brill et al., 2016). The initiation of myelination correlates with an increase in tubulin content (Fig.  
159 S2 G and H). Following BTX injection, microtubular mass on winner axons ('sin') decreased to ~60%, while  
160 competing axons ('din') were not affected (Fig. 4, F and G). This hints at the possibility that the delayed  
161 node formation following transmission block is due to reduced microtubular mass.

162 To manipulate axonal microtubules, we genetically deleted spastin, a microtubule-severing enzyme  
163 (spastin KO), where we confirmed a delay in axon branch removal (Fig. 4, H–J; Brill et al., 2016). Indeed,  
164 loss of spastin led to accelerated node formation in competing axons ('din') compared to wild-type (WT;  
165 Fig. 4 K). That this represented a cell autonomous effect in motor neurons was corroborated by inducing  
166 subset deletion in conditional spastin<sup>fl/fl</sup> x TdTomato reporter mice (Brill et al., 2016) using a cre-encoding  
167 adeno-associated virus (AAV9-CMV-iCre; Fig. S3). While we again found delayed axon remodeling (Brill et  
168 al., 2016), node formation was now accelerated on competing branches (Fig. S3 C), where TdTomato  
169 expression indicated spastin deletion. Overall, microtubular mass was increased in spastin-deleted  
170 terminal axon branches (Fig. 4, L and M; Brill et al., 2016), while axonal caliber was unaffected (Fig. S3 D),  
171 contrasting the increase in nodal formation specifically on competing branches. This suggests that the  
172 microtubular cytoskeleton is the limiting factor to initiate node formation in competing branches, but not  
173 on winner axons, perhaps arguing for a two-component system, where each can be limiting in different  
174 stages. Moreover, the divergent axon-glia differentiation outcomes of postsynaptic block vs. spastin  
175 deletion, which both delay remodeling, points to a mechanism that is blocked by ongoing activity-  
176 dependent competition, but can be overcome by increasing microtubular mass. As the microtubular  
177 cytoskeleton sustains axonal transport (which requires tracks and cargoes), we next tested if reducing  
178 anterograde transport would affect local initiation of axon-glia differentiation.

### 179 **Local axonal transport regulates terminal branch myelination during remodeling**

180 To reduce transport in motor neurons, we overexpressed the cargo-binding domain (CBD) of kinesin-1  
181 heavy chain (KHC), a key molecular motor driving anterograde transport (Hirokawa et al., 2009). This  
182 results in a dominant-negative mutant (KHC-CBD), which still binds cargoes, but lacks the motor domain  
183 and competes with endogenous kinesin-1, thus impairing transport of organelles and nodal components  
184 *in vitro* (Cai et al., 2005; Barry et al., 2014). To test the efficacy of this approach *in vivo*, we turned to

185 zebrafish as an easily accessible model for assaying effects of myelination-regulating signals (Czopka and  
186 Lyons, 2011). We used the Gal4/UAS system to transiently co-express UAS-GFP-KHC-CDB and UAS-  
187 mitoTagRFP-T in Rohon Beard sensory neurons, in which mitochondrial transport can be easily monitored  
188 (Plucińska et al., 2012). KHC-CBD overexpression in this system substantially reduced mitochondrial  
189 transport per minute (reduction from  $0.61 \pm 0.11$  in control to  $0.10 \pm 0.02$  in anterograde and  $0.28 \pm 0.04$   
190 to  $0.10 \pm 0.04$  in retrograde direction at 2 days post fertilization, dpf;  $P < 0.01$ , Mann-Whitney test,  $n \geq 4$   
191 zebrafish per group,  $\geq 4$  axons). We then analyzed spinal motor neurons, which start to be myelinated at  
192 3 dpf in zebrafish (D’Rozario et al., 2017). To down-regulate axonal transport while monitoring myelination  
193 progress, we expressed GFP-KHC-CBD or GFP alone under the neuronal *cntn1b* promoter in Tg(mbp:RFP)  
194 zebrafish, where all compacted internodes are fluorescently labelled by a membrane-targeted RFP (Auer  
195 et al., 2018; Fig. S4, A–E). On 6 dpf, axon length in Tg(mbp:RFP) zebrafish injected with *cntn1b*-GFP-KHC-  
196 CBD was similar to controls (Fig. S4 F), but strikingly, the myelinated axon length was only half compared  
197 to controls (Fig. S4 G). This supports the notion that PNS myelination depends on axonal transport.

198 We next probed whether this was true in murine motor axons during remodeling. In *Thy1*- $\beta$ 1-Nav-GFP  
199 animals, emerging  $\beta$ 1-Nav-GFP clusters correlate with higher anterograde particle transport in terminal  
200 branches (Fig. 5). Among all  $\beta$ 1-Nav-GFP positive branches, winner axons (‘sin’) had the highest  
201 anterograde transport rate (Fig. 5 B). Together, this is in line with our previous observation that  
202 microtubular mass correlates with node formation (Fig. S2 H). We then injected an AAV9 encoding KHC-  
203 CBD and iCre under control of the neuronal human synapsin promoter (AAV9-*hSyn*-iCre-p2a-KHC-CBD) into  
204 neonatal mice (Fig. 5 A). In AAV injected *Thy1*- $\beta$ 1-Nav-GFP x TdTomato reporter mice, we found a  
205 significant reduction in  $\beta$ 1-Nav-GFP anterograde transport, while retrograde was unaffected (Fig. 5 B).  
206 Notably, the onset of node formation was significantly delayed in reporter-positive branches compared to  
207 negative ones, which served as internal controls (Fig. 5, C and D). This points to a transport-delivered signal,  
208 which locally times the onset of axon-glia differentiation of terminal axon branches.

## 209 **Local disparity of pro-myelinating factors in terminal branches correlates with competition status**

210 Nrg1 type III is a candidate for a transported pro-myelination signal, as this signaling factor locally needs  
211 to reach a critical threshold to initiate axon-glia differentiation (Taveggia et al., 2005; Nave and Salzer,  
212 2006; Birchmeier and Nave, 2008; Velanac et al., 2012) by activating downstream effectors in SCs such as  
213 ERK1/2 and AKT (Ogata et al., 2004; Taveggia et al., 2005; Basak et al., 2015; Duregotti et al., 2015). To  
214 investigate Nrg1 type III function during axon remodeling, we crossed floxed Nrg1 type III (Velanac et al.,  
215 2012) to TdTomato reporter mice and injected neonates with AAV9-CMV-iCre (Fig. S3 E). As expected,  
216 myelination was severely impaired in TdTomato-positive branches compared to internal control axons (Fig.  
217 S3, F–H). We further tested if increased Nrg1 type III levels are sufficient to remove the competition-  
218 dependent block on myelination employing *Thy1*-Nrg1 type III-HA mice, where Nrg1 type III is tagged with  
219 hemagglutinin (Fig. 6 A and B). Here axon remodeling was transiently accelerated (Lee et al., 2016), and  
220 overall nodes form significantly earlier (Fig. 6 C–F; Velanac et al., 2012). Notably the myelination delay on  
221 competing ‘din’ branches was preserved, most likely reflecting the endogenous distribution of Nrg1 type  
222 III (Fig. 6 F).

223 To analyze local distribution of Nrg1 type III with single axon precision, we immunostained for the HA-  
224 tag and visualized SCs and axons (Fig. 7, A and B). Strikingly, we detected a higher HA-signal along winner  
225 ‘sin’ branches compared to competing ‘din’ axons, in line with differential trafficking regulated by  
226 competition-regulated cytoskeletal maturation (Fig. 7 C). Corroborating differential Nrg1 signaling, we  
227 measured significantly higher levels of activated forms of both ERK1/2 (pERK) and AKT (pAKT) surrounding  
228 winner ‘sin’ axons (Fig. 7, D–G). As myelination initiation on winner branches was reduced following  
229 neurotransmission blockade, HA-tagged Nrg1 type III and pERK signals also significantly decreased in ‘sin’  
230 branches (Fig. 7, H and I). Transgenic expression of Nrg1 type III did not change the density neither area of  
231 acetylcholine receptors (Fig. 7 J and K). Therefore, Nrg1 type III likely impacts myelination via its  
232 promyelinating effects rather than by modulating postsynaptic feedback (Velanac et al., 2012; Kamezaki



233 [et al., 2016](#)). Indeed, the only phenotype we observed at the endplate was a premature shape change of  
234 the acetylcholine receptor distribution in *Thy1-Nrg1* type III-HA mice compared to wild-type littermates  
235 ([Fig. 7 L](#); *cf.* [Lee et al., 2016](#)).

## 236 **DISCUSSION**

237 Taken together, our data suggest that during motor axon remodeling, a pro-myelination signal—such  
238 as *Nrg1*—paces branch myelination, which is locally limited by axonal transport and depends on local  
239 regulation of cytoskeletal integrity. Thus, axon-glia differentiation is delayed until competition resolves  
240 and the axonal cytoskeleton matures. Notably, according to this model, axon dismantling and myelination  
241 initiation both depend on mechanisms that regulate the microtubular cytoskeleton ([Brill et al., 2016](#)). This  
242 model also links anterograde transport to a signaling function, which previously was mostly considered for  
243 retrograde transport, *e.g.* delivering neurotrophic factors ([Je et al., 2012](#); [Yamashita, 2019](#)).

244 The intersection of axon remodeling and myelination is widespread ([Feinberg, 1982](#); [Bernstein and](#)  
245 [Lichtman, 1999](#); [Woo and Crowell, 2005](#); [Barres, 2008](#)) and might play a general role in the activity-  
246 dependent sculpting of efficient neuronal networks ([Luo and O’Leary, 2005](#); [Tapia and Lichtman, 2012](#); [de](#)  
247 [Hoz and Simons, 2015](#); [Chang et al., 2016](#)). The prevailing notion has been that myelination might  
248 terminate axonal plasticity by ‘cementing’ axons in place, thus contributing to closing the critical  
249 remodeling period ([Caroni and Schwab, 1988](#); [McGee et al., 2005](#); [Geoffroy and Zheng, 2014](#); [Kalish et al.,](#)  
250 [2020](#)). Indeed, we observed myelination initiation predominantly on winner axon branches (‘win’), *i.e.* after  
251 competition was resolved ([Fig. 1](#)). However, while determining the exact start and progression speed of  
252 myelination is technically challenging (thus we resorted to a ‘binary’ readout of *Caspr*+ vs. *Caspr*- branches),  
253 our observations clearly reveal that competition delayed myelination, but not *vice versa* ([Fig. 3](#)). A subset  
254 of competing axons still initiated myelination, but this did not convey a measurable advantage during  
255 competition, as no relationship between myelination and synaptic territory was apparent in our data. Even

256 some retreating axons were myelinated, including in cases where the likely competing branch was not (Fig.  
257 3 G). Still, in general myelination was prevented on axon branches that were fated for removal. As myelin  
258 is an extremely stable structure (Simons and Trotter, 2007; Hughes et al., 2018), which might be  
259 metabolically ‘expensive’ to build (Nave and Trapp, 2008; Harris and Attwell, 2012) and requires a  
260 dedicated mechanism for dismantling, it seems economical to delay myelination until pruning is resolved  
261 (McGee et al., 2005; Cheng and Carr, 2007). Thus our results support a view where myelin might act as  
262 participant in, but not as the terminator of circuit plasticity (Mount and Monje, 2017; but cf. Roche et al.,  
263 2014).

264 How do competing axon branches delay myelination? A number of cell biological features of such  
265 branches scale with its synaptic territory during competition, *e.g.* cytoskeletal stability, organelle transport  
266 or axon caliber (Keller-Peck et al., 2001; Brill et al., 2016). We can rule out the hypothesis that axon caliber  
267 dictates myelination onset, since axon caliber did not differ between myelinated and unmyelinated  
268 competing branches (Fig. 3 D; Goebbels et al., 2010), even though we found evidence that along a given  
269 branch, myelination has an impact on local diameter (Fig. 3 E). To further probe the mechanism that times  
270 myelination onset, we manipulated activity-driven competition (Buffelli et al., 2003) using local BTX  
271 injections (Fig. 4). This intervention is muscle-specific, therefore less likely to affect axon-SC  
272 communication, compared to blocking axonal action potential conduction or acetylcholine release  
273 (Misgeld et al., 2002; Lorenzetto et al., 2009). Notably, while there exists some cholinergic axon-Schwann  
274 cell communication, this typically involves BTX-insensitive receptors (Rousse and Robitaille, 2006). In the  
275 past, chronic blockade of neuromuscular transmission, *e.g.* in chicken embryos treated with curare, has  
276 been shown to result in AChR cluster fragmentation and axonal sprouting (*cf.* Loebet al., 2002; Loeb, 2003).  
277 Also, constitutive genetic ablation of choline acetyl transferase in motor axons leads to premature  
278 myelination in the phrenic nerve at birth (Misgeld et al., 2002). Still, these outcomes likely reflect the  
279 combination of presynaptic and sustained blockade, prone to elicit homeostatic compensation (Davis,

280 [2013](#)). Here, by using brief and local postsynaptic blockade, we found the expected delay in axon  
281 remodeling, but also a commensurate hiatus in myelination ([Fig. 4, D and E](#)). Thus, myelination onset  
282 appears to be part of the BTX-sensitive competition program, revealing an indirect role of  
283 neurotransmission in regulating the progress of PNS myelination (for the CNS, *cf.* [Stevens et al., 2002](#);  
284 [Gibson et al., 2014](#); [Krasnow et al., 2018](#)). Moreover, postsynaptic block induced a reduction in presynaptic  
285 microtubular mass on winner branches ([Fig. 4 F and G](#)), suggesting a silencing-induced delay in maturation,  
286 which chimes with a cytoskeletal mechanism of inducing myelination.

287 Indeed, in terminal axon branches, the microtubular cytoskeleton matures in parallel to an increase in  
288 synaptic territory ([Brill et al., 2016](#)). Accordingly, we observed that spastin deletion, which increased  
289 microtubular mass ([Fig. 4, L and M](#)), resulted in accelerated myelination specifically on competing branches,  
290 breaking the link between remodeling and myelination delay ([Fig. 4 K](#)). However, spastin deletion in winner  
291 branches did not affect the initiation of myelination. This suggests that the limiting factor in this setting  
292 could be cargo instead of track availability, as we reported previously for mitochondria ([Marahori, 2020](#)).  
293 Since microtubular content can locally regulate axonal transport ([Kapitein and Hoogenraad, 2015](#); [Roll-  
294 Mecak, 2019](#)), hinting that myelination might depend on branch-specific transport. To test this, we  
295 expressed a dominant-negative kinesin mutant *in vivo*, which affects anterograde organelle delivery ([Cai  
296 et al., 2005](#); [Barry et al., 2014](#)). In both zebrafish and mouse motor neurons this delayed myelination,  
297 despite the transport blockade being partial and short ([Fig. 5](#) and [Fig. S4](#)). Taken together, our data suggest  
298 that in competing branches, transport of pro-myelinating cargos is restricted by an immature and  
299 increasingly severed microtubular cytoskeleton ([Brill et al., 2016](#)).

300 Since our FRAP experiments suggest that nodal components are not limited in competing branches ([Fig.  
301 2](#)), we focused on Nrg1 type III as the putative factor determining myelination onset for the following  
302 reasons: Nrg1 signaling (1) is the master regulator of PNS myelination ([Birchmeier and Nave, 2008](#);  
303 [Grigoryan and Birchmeier, 2015](#)); (2) acts in a threshold-based manner ([Garratt et al., 2000](#); [Michailov et](#)

304 al., 2004; Taveggia et al., 2005; Nave and Salzer, 2006); (3) is steeply upregulated during the synapse  
305 remodeling period (Lee et al., 2016), while its axonal presence is limited (Velanac et al., 2012). Indeed, we  
306 demonstrated that HA-tagged Nrg1 accumulates faster on winner than on competing branches (Fig. 7 C),  
307 and down-stream pathways of Nrg1 signaling are preferentially activated in SCs around winner branches  
308 (Fig. 7, F and G). It would have further strengthened our argument if increased Nrg1 could be observed in  
309 spastin deleted axons. Likely due to chromosomal incompatibility of the Nrg1 transgene insertion site and  
310 the spastin locus, we tested the converse setting and showed that HA-tagged Nrg1 distribution and down-  
311 stream signaling decrease upon BTX blockade (Fig. 7, H and I). The fact that Nrg1 overexpression  
312 accelerates both myelination and synapse elimination (Fig. 6; Lee et al., 2016) further strengthens our  
313 conclusion that myelination *per se* does not terminate remodeling. However, it is technically challenging  
314 to disambiguate whether Nrg1 signals directly from the axonal surface to myelinating SCs, or through a  
315 more complicated feedback via muscle and/or terminal SCs. Yet we did not measure a difference in  
316 postsynaptic acetylcholine receptor density neither area between *Thy1*-Nrg1 type III-HA and wild-type  
317 littermates (Fig. 7), suggesting Nrg1 likely functions via its promyelinating effects, rather than modulating  
318 muscular depolarization.

319 In summary, our experiments reveal an intercellular signaling mechanism that regulates myelination on  
320 a branch-to-branch level in the developing PNS. The extent of branch-specific Nrg1 accumulation, and  
321 hence the strength of the pro-myelination signal, is regulated by the axonal cytoskeleton as a spatially-  
322 resolved signaling hub (Janke, 2014). A similar local regulation between neuronal remodeling and  
323 myelination can be relevant in many developing neural circuits, *e.g.* certain cortical axon types are  
324 myelinated in a highly local fashion (Tomassy et al., 2014; Micheva et al., 2016). Moreover, when disturbed,  
325 such signaling could contribute to the disrupted timing of developmental events characteristic of some  
326 neuropsychiatric disorders, where axonal transport, neuronal remodeling and myelination all show subtle  
327 defects (Coleman and Perry, 2002; Luo and O'Leary, 2005; Mei and Nave, 2014).

## 328 MATERIALS AND METHODS

### 329 Mouse lines and husbandry

330 In all experiments, mice from both sexes were included. Animals were housed in individually ventilated  
331 cages with food and water ad libitum. All animal experiments were performed in accordance with the  
332 regulations by the local authorities, e.g. Government of Upper Bavaria. Experimental animals were kept  
333 together with littermates.

### 334 Experimental mice and genotyping

335 For labeling axons we used *Thy1*-XFP mice, which express OFP (*Thy1*-OFP3; Brill et al., 2011), YFP (*Thy1*-  
336 YFP16; Feng et al., 2000), or membrane-RFP (*Thy1*-Brainbow-1.1 line M; Livet et al., 2007) under the  
337 control of the *Thy1*-promotor (Feng et al., 2000). For FRAP experiments and node visualization in living  
338 explants, we used *Thy1*-Caspr-GFP (Brivio et al., 2017) and *Thy1*- $\beta$ 1-Nav-GFP transgenic mice (generated  
339 for this study, see below) crossed to *Thy1*-XFP mice. Synaptic territory of competing axonal branches was  
340 defined in *Thy1*-Brainbow-1.1 line M mice crossed to Cre-expressing lines: CAG-CreERT (gift from Dr. J.  
341 Livet, Institut de la Vision, Paris, France) or *ChAT*-IRES-Cre (Jackson, #6410; Rossi et al., 2011), which leads  
342 to individual fluorescent color combinations of membrane-targeted RFP, YFP, and CFP. For the crossing  
343 involving CAG-CreERT, 20 $\mu$ l of 1.5 mg/ml tamoxifen was subcutaneously injected on postnatal day (P) 3 to  
344 induce expression. Delayed synapse elimination was analyzed in spastin knock-out (KO) mice (Brill et al.,  
345 2016) or spastin floxed (<sup>fl/fl</sup>) mice bred to *ROSA*-CAG-TdTomato or YFP reporter mice (Jackson, #7914,  
346 #7903; Madisen et al., 2010) injected with AAV9-CMV-iCre (provided by Dr. Engelhardt; Brill et al., 2016).  
347 Effects of transport modulation on myelin and nodal development was observed in TdTomato reporter  
348 mice crossed to *Thy1*- $\beta$ 1-Nav-GFP or *ROSA*-CAG-YFP mice injected with AAV9-hSyn-iCre-p2a-KHC-CBD.  
349 Precocious myelination was investigated in *Thy1*-Nrg1 type III-HA mice (“HANI”, Velanac et al., 2012). To

350 investigate delayed nodal development, we injected AAV9-CMV-iCre into conditional Neuregulin knock-  
351 out mice (Nrg1 type III<sup>fl/fl</sup>; [Velanac et al., 2012](#)) crossbred with ROSA-CAG-TdTomato reporter. To visualize  
352 SCs, we used *Plp*-GFP transgenic mice ([Mallon et al., 2002](#)). All experiments on ROSA-CAG-TdTomato  
353 reporter mice were performed in homozygous animals.

354 Genomic DNA was extracted from tail biopsies using a one-step lysis (lysis buffer in mM: 67 Tris pH 8.8,  
355 16.6 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6.5 MgCl<sub>2</sub>, 5 β-mercaptoethanol, 10% Triton and 50 µg/ml Proteinase K; incubation at 55°C  
356 for 5 hours, followed by inactivation step 5 min at 95°C). PCR was performed with GoTaq Green Master  
357 Mix (Promega, #M7121) following a standard protocol, then DNA was separated on a 1.5 - 2 % agarose gel.  
358 Genotyping primers and expected products are listed in Table S1.

### 359 **Generation of Thy1-β1-Nav-GFP transgenic mice**

360 Transgenic mice expressing the beta 1 subunit of the sodium channel (β1-Nav) fused to GFP at the C-  
361 terminus under the control of the *Thy1.2* promoter ([Caroni, 1997](#)) were generated by pronuclear injection.  
362 The β1-Nav-GFP cDNA ([McEwen et al., 2009](#)) was cloned into the blunted XhoI site of the pTSC21k vector  
363 ([Lüthi et al., 1997](#)), released using Not I ([Zonta et al., 2011](#)), and used for pronuclear injection ([Sherman](#)  
364 [and Brophy, 2000](#)).

### 365 **FRAP experiments and Caspr-GFP trafficking in nerve-muscle explant**

366 Nerve-muscle explants from the thorax including the *triangularis sterni* muscle were prepared from  
367 young (postnatal day 7 - 14) or adult mice (6 weeks; [Kerschensteiner et al., 2008](#); [Brill et al., 2011, 2016](#)).  
368 The rib cage was isolated from euthanized animals, and the skin and pectoral muscles over the rib cage  
369 were removed. The diaphragm was cut and the thorax was released by cutting the ribs close to the  
370 vertebral column. The dissection was continued in oxygenated precooled Ringer's solution (in mM: 125  
371 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>, 20 glucose, oxygenated with 95 % O<sub>2</sub>/ 5 % CO<sub>2</sub>)  
372 in a 10 cm dish and remnants of muscles, thymus, pleura and lung were removed. The clean nerve-muscle

373 explant was pinned onto a Sylgard-coated 3.5 cm dish, superfused with oxygenated Ringer's solution, using  
374 shortened insect pins (0.25 mm Fine Science Tools, 26001-25), exposing the *triangularis sterni* muscle, the  
375 intercostal nerve and terminal motor neuron branches. During imaging the explant was kept at 31-33°C  
376 with a heating ring connected to an automatic temperature controller (TC-344C, Warner Instruments) and  
377 steadily perfused with Ringer's solution.

378 Trafficking measurements of  $\beta$ 1-Nav-GFP particles in the terminal branches were performed with an  
379 Olympus BX51WI epifluorescence microscope equipped with  $\times$ 20/0.5 N.A. and  $\times$ 100/1.0 N.A. water-  
380 immersion objectives, an automated filter wheel (Lambda 10-3, Sutter Instrument), a CCD camera  
381 (CoolSnap HQ2, Visitron Systems), and a GFP ET filter set (AHF Analysentechnik). All devices were  
382 controlled by  $\mu$ Manager 1.4 ([Edelstein et al., 2014](#)). Per movie, we acquired 200 images at 1 Hz using an  
383 exposure time of 500 ms. Total imaging time on explants was restricted to maximum two hours, except  
384 for in FRAP analysis (below).

385 For FRAP analysis, we used *Thy1-Caspr-GFP* and *Thy1- $\beta$ 1-Nav-GFP* mice and the same setup as  
386 described above for transport measurements. The laser (473 nm, DL-473, Rapp OptoElectronic) for  
387 photobleaching was manually focused on a labeled node of Ranvier ( $\sim$ 5  $\mu$ m<sup>2</sup>) and the sample was bleached  
388 for 1 - 3 seconds. We performed FRAP on branches with heminodes during development, since fully  
389 developed nodes are rare at the investigated age. In adults, all measurements were performed on fully  
390 developed nodes. The GFP signal was imaged with 800 ms exposure time before and immediately after  
391 photobleaching with a GFP/mCherry dualband ET filter set (AHF Analysentechnik), then in one-hour  
392 intervals for three hours with 800 ms exposure time.

### 393 **Mouse immunofluorescence and confocal microscopy**

394 The thorax was fixed in 4 % paraformaldehyde (PFA) for one hour in 0.1 M phosphate buffer (PB) on ice  
395 and the *triangularis* muscle was dissected and extracted ([Brill et al., 2011](#)). For HA staining, the sample was

396 additionally treated for 1 h at 37°C in 5 % CHAPS. The fixed thorax was incubated overnight (or 72 h for HA,  
397 pERK and pAKT) at 4°C in the respective primary antibodies diluted in blocking solution (5 % BSA, 0.5 %  
398 Triton X-100 in 0.1 M PB). To label postsynaptic nicotinic acetylcholine receptors Alexa 488-, Alexa 594-,  
399 Alexa 647- or biotin-conjugated to  $\alpha$ -bungarotoxin (BTX; Invitrogen, B13422, B13423, B35450, B1196; 50  
400  $\mu$ g/ml, 1:50) was added to the primary antibody mixture. The following primary antibodies were used in  
401 this study: anti- $\beta$ III-tubulin conjugated to Alexa 488 (BioLegend, AB\_2562669; mouse IgG2a, 1:200), Alexa  
402 555 (BD Pharmingen, #560339; mouse monoclonal, 1:200), Alexa 647 (BioLegend, AB\_2563609; mouse  
403 IgG2a, 1:200). For labeling of nodal components antibodies against Caspr (Abcam, AB\_869934, polyclonal  
404 rabbit; 1 mg/ml 1:400), MPZ (Aves Labs combined chicken IgY, 200  $\mu$ g/ml, 1:200), CNTN2 (R&D Systems,  
405 AB\_2044647; polyclonal goat IgG, 1:200), pan Nav subunit  $\alpha$  (Abgent, AG1392; polyclonal rabbit, 1.0 mg/ml,  
406 1:400). HA was stained with anti HA-tag antibody (Cell Signaling, AB\_1549585; rabbit, 1:50) and  
407 phosphorylated ERK1/2 was stained with anti-phospho-p44/42 MAPK (Cell Signaling, AB\_331646; rabbit,  
408 1:200). Here we used the tyramide signal amplification (TSA Cyanine 3 System, Perkin Elmer, AB\_2572409).  
409 Muscles were washed in 0.1 M PB, incubated for one hour at room temperature with corresponding  
410 secondary antibodies coupled to Alexa 488, Alexa 594 or Alexa 647 (Invitrogen, rabbit: #A-11070, #A-11072,  
411 #A-21246, #A-32790; mouse: #A-11005; chicken: #A-11042; #A-21449; goat: #A-11058) and washed again  
412 in 0.1 M PB. Muscles were mounted in Vectashield (Vector Laboratories) or Fluoromont-G (Southern  
413 Biotech) and image stacks were recorded using a confocal microscope (Olympus FV1000) equipped with  
414 x20/0.8 N.A. and x60/1.42 N.A. oil-immersion objectives (Olympus).

#### 415 **Generation of recombinant DNA**

416 In order to generate the pTREK1-*hSyn*-iCre-p2a-KHC-CBD construct for the AAV9-*hSyn*-iCre-p2a-KHC-  
417 CBD production, we used the Gibson Assembly Master Mix (NEB). We recombined fragments from pEGFP-  
418 C1-KHC-CBD (Cai et al., 2005), the dsCMV-iCre and the pAAV-*hSyn*-DIO-HA-hM3D(Gq)-IRES-mCitrine  
419 plasmid (addgene #50454) and the p2a sequence. The produced amplicon was ligated into the single-



420 stranded AAV backbone plasmid pTREK1. The following oligonucleotides were used:  
421 AGTACTTAATACGACTCACTATAGGATGGTGCCCAAGAAG, TCCACGTCGCCGGCCTGCTTCAGCAGGGAGAAGT-  
422 TGGTGGCGTCCCATCCTCGA, TGCTGAAGCAGGCCGGCGACGTGGAGGAGAAACCCCGGCCCCAGTGCTGAGATT-  
423 GATTCT, and ATCATGTCTGGATCCTCGATAGTTTAAACTTACACTTGTTTGCTC.

424 For zebrafish injections, we generated pDestTol2CG2\_UAS:GFP-KHC-CBD-polyA and  
425 pTol2\_cntn1b:KHC-CBD-GFP vectors using the Gateway system (Thermo Fisher). To produce the middle  
426 entry clone pME\_GFP-KHC-CBD, the GFP-KHC-CBD sequence was amplified from the template plasmid  
427 (Cai et al., 2005). The PCR product was then recombined into the vector pDONR221 using BP clonase  
428 (Thermo Fisher). The final expression constructs pTol2\_UAS:GFP-KHC-CBD and pTol2\_cntn1b:GFP-KHC-  
429 CBD were then generated in multisite LR recombination reactions with the entry clones, p5E\_UAS,  
430 p5E\_cntn1b, pME\_GFP-KHC-CBD, p3E\_pA and pDestTol2\_pA of the Tol2Kit (Kwan et al., 2007).

#### 431 **Generation of AAV9 (adeno-associated virus serotype 9)**

432 HEK293-T cells were grown in 10-tray Cell Factories (Thermo Scientific) using Dulbecco's modified  
433 essential medium (Gibco) with 10 % fetal bovine serum (Gibco) and 1 % penicillin/streptomycin (Gibco).  
434 The cells were split into the Cell Factories 24 h prior to transfection to reach 80 % confluence at the time  
435 of transfection. Then, 420 µg of the pTREK1-*hSyn*-iCre-p2a-KHC-CBD plasmid and 1.5 mg of the helper  
436 plasmid (pDP9rs, kindly provided by Roger Hajjar) were introduced into the HEK293-T cells using  
437 polyethylenimine (Polysciences). Three days later the cells were harvested, lysed, benzonase-treated and  
438 the virus was isolated by ultracentrifugation through an iodixanol density gradient (Optiprep, Progen).  
439 Ringer lactate buffer (Braun) was used to replace iodixanol with the help of Vivaspin 20 columns, MWCO  
440 100000 PES (Sartorius). Two 10-tray Cell Factories were pooled and concentrated to a total volume of 500  
441 µl. AAV9 titers were determined by real-time PCR using SYBR Green Master Mix (Roche). Titers in the range  
442 of  $1 \times 10^{14}$  viral genome copies per milliliter (vg/ml) were acquired.

### 443 Neonatal AAV9 or $\alpha$ -BTX-injection

444 AAV9 was injected into neonatal pups according to previously published protocol ([Passini and Wolfe,](#)  
445 [2001](#)). In short, P3 pups were anaesthetized with isoflurane (Abbott) and injected with 3  $\mu$ l AAV9-CMV-  
446 iCre or AAV9-hSyn-iCre-p2A-KHC particles into the right lateral ventricle at a rate of 30 nl/s using a fine  
447 glass pipette (3.5" Drummond #3-000-203-G/X) attached to a nanoliter injector (Micro4 MicroSyringe  
448 Pump Controller connected with Nanoliter 2000, World Precision Instruments). All surgeries were  
449 conducted under ultrasound guidance (Vevo1100 Imaging System, with a Microscan MS550D transducer,  
450 Visualsonics). 0.05 % (wt/vol) trypan blue was added to the viral solution for visualizing the filling of the  
451 injected ventricles. Only whole litters were injected, and pups were allowed to recover on a heating mat  
452 before the litter was returned to their mother into the home cage and sacrificed on P9 for  
453 immunohistochemistry. To monitor Cre-mediated recombination, mice carried in addition to the  
454 respective genes (spastin or Nrg1 type III conditional knock-out), two TdTomato or YFP reporter alleles  
455 (homozygous), which resulted in robust expression of the reporter in a subset of motor neurons.

456 Injection of  $\alpha$ -BTX on P 7 was administered in a similar manner, only the needle was inserted laterally  
457 under the skin of the right thorax, and 1  $\mu$ l of a 50 mg/ $\mu$ l Alexa 488- or 594-conjugated BTX solution  
458 (Invitrogen, B13422, B13423) was injected. The contralateral (left) *triangularis sterni* muscle was  
459 unaffected and used as control. The injected pups were viable and active after the treatment, and not  
460 distinguishable from untreated controls. Ipsi- and contralateral *triangularis sterni* muscle were then *post*  
461 *hoc* stained with Alexa 594- or 488-conjugated BTX respectively, resulting in complementing stainings for  
462 blocked and unblocked AChRs. We confirmed a substantial degree of persisting blockade at P9 ( $11.9 \pm 4.5$   
463 fold change of BTX staining on injected vs. non-injected side,  $n \geq 50$  NMJs in 5 mice) and the absence of  
464 denervation ( $> 100$  NMJs per mouse,  $n = 3$  mice).

## 465 Zebrafish injection, immunostaining and confocal imaging

466 Fertilized Tg(mbp:RFP) eggs (Auer et al., 2018) at the one cell stage were pressure microinjected with  
467 1 nl solution containing 20 - 40 ng/ $\mu$ l plasmid DNA (*cntn1b*:GFP-KHC-CBD or control *cntn1b*:GFP; Auer et  
468 al., 2018) and 25 - 50 ng/ $\mu$ l transposase mRNA. For immunohistochemistry, larval zebrafish at 6 dpf were  
469 euthanized with 4 mg/ml MS-222 (PHARMAQ) and then fixed overnight in 4 % PFA in 0.1 M PB. After  
470 fixation, the samples were washed three times in PBS, 0.1 % Tween20 and then immersed for 2 hours at  
471 room temperature in blocking solution (5 % BSA, 0.5 % Triton X-100 in 0.1 M PB), then incubated in primary  
472 antibody against  $\alpha$ -tubulin (Sigma-Aldrich, #00020911, mouse, 1:200) at 4° C for 48 hours in blocking  
473 solution. Samples were washed and incubated in secondary antibody conjugated to Alexa 647 (Invitrogen,  
474 goat-anti-mouse #A-28181) overnight at 4°C (Hunter et al., 2011). Samples were washed again and  
475 embedded in Vectashield (Vector Laboratories). Image stacks were recorded using a confocal microscope  
476 (Olympus FV1000) equipped with a x20/0.8 N.A. oil-immersion objective.

477 To label Rohon-Beard neurons, fertilized embryos from wild-type fish were co-injected with a sensory  
478 neuron-specific Gal4 driver construct (containing enhancer elements from *isl1*; Sagasti et al.,  
479 2005) together with UAS:KHC-CBD-GFP and UAS:mitoTagRFP-T plasmids (each at 5 ng/ $\mu$ l).  
480 Alternatively, UAS:KHC-CBD-GFP and UAS:mitoTagRFP-T plasmids were co-injected into fertilized eggs  
481 from the *isl2b*:Gal4 line (Fredj et al., 2010). At 2 dpf, embryos were anesthetized using 0.2 mg/ml MS-222  
482 (PHARMAQ) and embedded in UltraPure Low Melting Point Agarose (Thermo Fisher) on a glass coverslip.  
483 After selecting double labeled Rohon-Beard neurons, mitochondrial transport was imaged for at least 50  
484 min in the stem axon using the wide-field microscope configured as in the FRAP experiments. We acquired  
485 movies with an imaging frequency of 2 Hz and an exposure time between 200 and 500 ms for each fish  
486 (Plucińska et al., 2012).

## 487 **Image processing/representation and quantification**

488       Innervation patterns was determined by counting the number of innervating terminal branches ending  
489 on each BTX stained neuromuscular junction (NMJ) in ImageJ/Fiji ([Schindelin et al., 2012](#)). The myelination  
490 status of a terminal branch (axon from last bifurcation until NMJ) was determined by any presence of  
491 clustered markers of nodal or internodal differentiation (Caspr, CNTN2, MPZ, Nav). Immunostaining on  
492 branchpoints were excluded due to difficulties to discern from the more prominent nodal structures on  
493 the stem axon. Axon diameter was determined by measuring the area of the entire terminal branch, then  
494 divided by the length of the branch, resulting in an averaged axon diameter. We verified the precision of  
495 our axonal diameter measurement by comparison with other methods to determine axonal diameter (*e.g.*  
496 averaged multi-site measurements; determination of smallest diameter *etc.*), and found no significant  
497 difference between Caspr-positive and negative axons in measured caliber with the different approaches  
498 we tested.

499       Tubulin content of axons was determined by manually placing regions of interest in a single optical  
500 section within an axon, and the mean grey values were averaged for each channel. We have previously  
501 established that immunohistochemically determined tubulin content correlates linearly with microtubule  
502 density as measured by electron microscopy, once corrected for an offset likely representing non-  
503 polymerized tubulin ([Brill et al., 2016](#)).

504       For FRAP analysis, in focus images were manually aligned and the intensity of the bleached area was  
505 measured with the polygon tool. The background intensity was measured in a dark and even area, and  
506 another GFP-positive paranode in the same field of view was used as control to correct for the recovery  
507 rate.

508       For zebrafish myelination, the motor axon length was determined using the segmented line tool based  
509 on  $\alpha$ -tubulin staining, and the length of the myelinated stretch is likewise determined based on mbp:RFP  
510 fluorescence.

511 Colocalization of GFP-positive Nav puncta and antibody staining was analyzed in single optical sections  
512 of unprocessed images.

513 To determine transport rates of mitochondria or  $\beta$ 1-Nav-GFP particles travelling along the axons, we  
514 counted the number of fluorescent particles passing through a region in focus of the axon quantified.

515 For image representation, maximum intensity projections were generated from confocal image stacks  
516 with ImageJ/Fiji, then further processed in Adobe Photoshop where channels were adjusted individually.  
517 For better visibility of dim structures gamma was adjusted in images that only represent morphological  
518 detail; no gamma adjustment was performed in quantitative images (all panels in [Fig. 2, A and C](#); [Fig. 4, F](#)  
519 [and L](#); [Fig 6, A and B](#); [Fig. 7, B and E](#); [Fig. S2, G](#)).

520 All analysis was performed with the experimenter blinded to the treatment or genotypes during  
521 imaging and scoring.

## 522 **Statistical Analysis**

523 Statistical tests were performed using the GraphPad PRISM software. Statistical significance was  
524 determined using the Mann-Whitney test (non-parametric test for two groups), following the Kruskal-  
525 Wallis test with post hoc Dunn's multiple comparisons test (non-parametric test for three or more groups)  
526 respectively. Unpaired t-test was used when the data set passed the D'Agostino & Pearson normality test.  
527 The  $\chi^2$  test was used for comparing expected frequencies between groups, and the p-value calculated  
528 from the test was shown. Group sizes were determined using experience values from prior studies (*e.g.*  
529 [Brill et al., 2016](#); [Plucińska et al., 2012](#)).  $P < 0.05$  was considered to be significant, and indicated by "\*\*";  $P$   
530  $< 0.01$  by "\*\*\*";  $P < 0.001$  by "\*\*\*\*"; and  $P < 0.0001$  by "\*\*\*\*\*". Bar graphs show mean + standard error of  
531 the mean. Violin plots depict median and quartiles excluding the outliers, which were identified with  
532 Tukey's test ([Fig. 2, B and D](#); [Fig. 5, B](#); [Fig. S2, H](#); [Fig. S3, D](#)).

## 533 **SUPPLEMENTAL MATERIAL**

534 Supplemental information includes 4 supplemental figures and one supplemental table.

## 535 **AUTHOR CONTRIBUTIONS**

536 Conceptualization, M.W., T.M. and M.S.B.; Investigation, M.W., T.K., G.P. and Y.X.; Methodology, D.L.S.,  
537 P.J.B.; Resources, P.A., S.E., M.H.S., P.B., D.S., M.K. and T.C.; Writing –Original Draft, M.W. and M.S.B.;  
538 Writing –Review & Editing, all authors; Supervision, T.M. and M.S.B.; Funding Acquisition, T.M.

## 539 **ACKNOWLEDGEMENTS**

540 We would like to thank M. and N. Budak, K. Wullimann and Y. Hufnagel for excellent technical  
541 assistance. We thank Dr. L. Godinho for critically reading the manuscript. We are grateful to Dr. J. Livet for  
542 the generous gift of *CAG-CreERT* mice and Dr. J. Trimmer for the Caspr antibody. Work in T.M.'s lab is  
543 supported by the Deutsche Forschungsgemeinschaft (DFG; CIPSM EXC114, CRC870, FOR 2879, Mi 694/7-1  
544 and 8-1, TRR274 - TRR 274/1 2020; ID 408885537, projects B03, C02), the European Research Council  
545 (FP/2007-2013; ERC Grant Agreement n. 616791), the Gemeinnützige Hertie Stiftung, the German-Israeli  
546 Foundation (I-1200-237.1/2012), the German Center for Neurodegenerative Diseases (DZNE) and  
547 Technische Universität München's (TUM) Institute for Advanced Studies. T.M. and M.S.B. were further  
548 funded by the DFG-funded Excellence Cluster SyNergy (EXC 2145 – ID 390857198). M.S.B. is recipient of a  
549 DFG research grant (LE 4610/1-1). M.W. received support from the TUM PhD program 'Medical Life  
550 Sciences and Technology'. D.L.S. and P.J.B. were supported by an award from the Wellcome Trust (Grant  
551 No. 107008). The authors declare no competing financial interests.

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863

864 **ABBREVIATIONS**

- 865 AAV: adeno-associated virus
- 866 AChR: acetylcholine receptor
- 867 BTX: bungarotoxin
- 868 Caspr1: contactin-associated protein 1
- 869 CBD: cargo-binding domain
- 870 CNS: central nervous system
- 871 CNTN2: contactin-2
- 872 din: doubly innervated NMJ
- 873 dpf: days post fertilization
- 874 FRAP: fluorescence recovery after photobleaching
- 875 KHC: kinesin-1 heavy chain
- 876 KO: knock-out
- 877 Nav: voltage-gated sodium channel
- 878 NMJ: neuromuscular junction
- 879 MPZ: myelin protein zero
- 880 Nrg1: Neuregulin-1
- 881 pERK: phosphorylated form of extracellular signal–regulated kinase 1/2
- 882 PNS: peripheral nervous system
- 883 P: postnatal day
- 884 SC: Schwann cell
- 885 sin: singly innervated NMJ

886 **FIGURE LEGENDS**

887 **Figure 1 | Myelination coincides with axon remodeling during the second postnatal week.**

888 **(A)** Schematic of thoracic nerve-muscle explant indicating anatomy of motor axons (dark gray). ‘Stem axon’  
889 in intercostal nerve; ‘soma’, motor neuron cell body in spinal cord; triangularis sterni muscle (pink);  
890 sternum and ribs (light gray). Boxed area of ‘terminal branches’ is schematized in more detail in (B). **(B)**  
891 Schematic of terminal branches of motor neurons (dark gray), postsynaptic acetylcholine receptors (‘NMJ’;  
892 blue). Din, doubly innervated NMJ, black arrowheads point to two ‘competing branches’ leading to the  
893 same NMJ; sin, singly innervated NMJ, ‘winner branch’. Regions of nodes of Ranvier: paranodes (green),  
894 node (red), juxtaparanodes (cyan). Schwann cells myelinate axons in internodal regions (magenta). **(C)** P7,  
895 9, 11 *triangularis sterni* muscles of *Thy1-YFP16* mice (axon, white), immunostained for Caspr (green),  
896 postsynaptic acetylcholine receptors (BTX, blue). Inset shows emerging paranodal Caspr cluster at P9.  
897 Corresponding schematics to the right, axons (gray) and Caspr-positive paranodes (green). Black  
898 arrowheads point to two axons leading to the same NMJ. **(D)** Quantification of the percentage of doubly  
899 innervated NMJs at P7, P9, and P11 ( $n \geq 5$  mice,  $\geq 100$  NMJs per animal, gray) and the percentage of Caspr-  
900 positive terminal branches among singly innervated NMJs ( $n \geq 5$  mice,  $\geq 100$  NMJs per animal, gray) and  
901 the percentage of Caspr-positive terminal branches among singly innervated NMJs ( $n \geq 7$  mice,  $\geq 30$   
902 branches per animal, green). **(E)** Nodes of Ranvier and myelin components: Immunostaining for Caspr  
903 (green, paranode), Nav (red, nodal region), CNTN2 (cyan, juxtaparanode) and MPZ (magenta, myelin) in  
904 single terminal axon branches of *Thy1-XFP* mice (axons, white). **(F)** Quantification of the percentage of  
905 myelin initiation on winner (singly innervating, ‘sin’) or competing (doubly innervating, ‘din’) terminal axon  
906 branches for Caspr (green), Nav (red), CNTN2 (cyan), or MPZ (magenta;  $n \geq 5$  mice per group,  $\geq 50$   
907 branches). Data, mean  $\pm$  SEM in (D), mean + SEM in (F). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Mann-Whitney test. Scale  
908 bars, 10  $\mu$ m in (C) overview, 2  $\mu$ m in (C) inset and (E).



909 **Figure 2| Nodes on competing branches are immature compared to those on winner branches.**

910 **(A)** Live image of motor axons in P11 *Thy1-Caspr-GFP* (green) x *Thy1-OFP3* (axon, white) nerve-muscle  
911 explant; dashed boxes indicate location of control (Ctrl) and photobleached (FRAP) nodes. Images on the  
912 right are taken before, directly after photobleaching, and 3 hours (h) later. Fire look-up table on the right.  
913 **(B)** Quantification of Caspr-GFP recovery rate comparing winner branches (sin) of different developmental  
914 ages (6 weeks, wk vs. P9-11) and different competition status at the same developmental age (P9-11 sin,  
915 din, stem;  $n \geq 13$  axons,  $\geq 10$  mice per group). **(C)** Live image of axon branches in P11 *Thy1- $\beta$ 1-Nav-GFP*  
916 (red) x *Thy1-OFP3* (axon, white) nerve-muscle explant; dashed boxes and images on right as in (A).  
917 **(D)** Quantification of  $\beta$ 1-Nav-GFP recovery rate as in (B;  $n \geq 9$  axons,  $\geq 5$  mice per group). ‘Din’, doubly  
918 innervating competing branch; ‘sin’, singly innervating winner branch. Data, mean + SEM. \*,  $P < 0.05$ ; \*\*,  
919  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ , Mann-Whitney test; outliers identified with Tukey’s test. Scale bars, 10  $\mu$ m in  
920 (A) and (C) overview, 2  $\mu$ m in insets.

921  
922 **Figure 3| Myelination of competing branches neither biases competition nor reflects axon**  
923 **diameter.**

924 **(A)** Image of a fixed *triangularis sterni* muscle of a *ChAT-IRES-Cre* x *Thy1-Brainbow-1.1* mouse. Motor units  
925 labeled with distinct fluorescence (axon, orange and white) and immunostained for Caspr (green);  
926 arrowheads point to competing branches and asterisk marks a pruning axon. Inset shows enlarged dashed  
927 box with emerging dotted and more mature paranodal structures. **(B)** Quantification of Caspr  
928 immunostaining vs. synaptic territory of competing branches ( $n \geq 78$  axons per group from a total of 69  
929 mice). **(C)** Graph of measured myelination patterns on paired competing branches vs. the calculated  
930 distribution assuming random myelin initiation. Winner is an axon branch  $> 50\%$  territory, loser  $\leq 50\%$   
931 territory. **(D)** Quantification of an axon’s diameter vs. its synaptic territory in axon branches either with  
932 (green) or without Caspr-immunostaining (gray;  $n \geq 10$  axons,  $\geq 7$  mice per group). **(E)** Quantification of

933 the diameter of stretches on retreating axons with (magenta) or without MPZ-immunostaining (gray;  $n \geq$   
934 8 axons,  $\geq 4$  mice per group) **(F, G)** Images of *Thy1*-XFP terminal branches (axons, white) stained for Caspr  
935 (green) and MPZ (magenta). Schematics to the right depict (F) a myelinated winning branch (black) vs. a  
936 pruning axon (gray; asterisk) without nodes; and (G) a rare example of a myelinated retreating branch  
937 (gray; asterisk) and its winning MPZ- and Caspr-negative competitor (black). ‘Rebu’, retraction bulbs; ‘sin’,  
938 winner axons. Data, mean  $\pm$  SEM. \*\*,  $P < 0.01$ , Mann-Whitney test. Scale bars, 10  $\mu$ m in (A), (F) and (G).

939  
940 **Figure 4 | Neurotransmission and spastin differentially affect myelination and microtubular**  
941 **mass.**

942 **(A)** Schematic of experimental design. *Thy1*-YFP16 mice were unilaterally injected with BTX (‘BTX inj’,  
943 orange) into the thoracic wall at P7, resulting in local blockade of acetylcholine receptors. Fixed ipsi- and  
944 contralateral muscles are post-hoc stained at P9 with BTX (blue) and immunostained for Caspr (green).  
945 **(B)** Contralateral control muscle, and **(C)** ipsilateral BTX-injected muscle; axons (*Thy1*-YFP16, white), Caspr  
946 immunostaining (green), *post hoc* stained BTX (blue), injected BTX (orange). Schematics below depict  
947 motor neurons (gray) and Caspr paranodes (green); black arrowheads point to two competing axons  
948 leading to the same NMJ. **(D)** Quantification of doubly innervated NMJs at P9 following BTX-injection ( $n =$   
949 8 mice,  $\geq 50$  axons per animal). **(E)** Quantification of Caspr-positive competing (‘din’) and winner (‘sin’)  
950 axon branches from BTX-injected muscles vs. controls ( $n = 6$  mice,  $\geq 32$  axons per side of animal). **(F)** Images  
951 of competing (‘din’) and winner (‘sin’) terminal branches following BTX injection (‘BTX inj’, orange) and  
952 post-hoc staining at P9 with BTX (blue) and  $\beta$ III-tubulin (white). **(G)** Quantification of  $\beta$ III-tubulin intensity  
953 (x-fold normalized to *Thy1*-YFP16;  $n \geq 5$  mice,  $n \geq 20$  axons per side of animal). **(H, I)** P9 *triangularis sterni*  
954 muscle of (H) littermate wild-type control (WT) and (I) spastin KO mouse. Axons immunostained for Caspr  
955 (green) and  $\beta$ III-tubulin (white). Corresponding schematics below, axons (gray) and Caspr-positive  
956 paranodes (green). Black arrowheads point to two axons innervating the same NMJ. **(J)** Quantification of

957 doubly innervated NMJs in P9 spastin KO animals compared to WT littermates ( $n \geq 5$  mice,  $n \geq 70$  axons  
958 per animal). **(K)** Quantification of Caspr-positive terminal branches in P9 spastin KO compared to WT  
959 littermates ( $n \geq 7$  mice,  $n \geq 33$  axons per animal). **(L)** Images of competing ('din') and winner ('sin') terminal  
960 branches in spastin WT and KO littermates, immunostained for  $\beta$ III-tubulin (white). **(M)** Quantification of  
961  $\beta$ III-tubulin intensity (x-fold normalized to *Thy1*-YFP16) in spastin KO vs. WT littermates ( $n \geq 5$  mice,  $n \geq 13$   
962 axons per animal). 'Din', competing axons; 'sin', winner axons. Data, mean + SEM. Mann-Whitney test \*,  $P$   
963  $< 0.05$ ; \*\*,  $P < 0.01$ . Scale bars, 10  $\mu\text{m}$  in (B), (C), (H) and (I), 5  $\mu\text{m}$  in (F) and (L).

964

### 965 **Figure 5 | Axonal transport limits myelination onset in terminal motor axon branches.**

966 **(A)** Schematic of experimental design. AAV9-*hSyn*-iCre-p2a-KHC-CBD was injected at P2 into the 3<sup>rd</sup>  
967 ventricle of YFP reporter mice. Muscles were analyzed at P9. **(B)** Quantification of axonal GFP particle  
968 transport in  $\beta$ 1-Nav-GFP animals ( $n \geq 16$  axons,  $\geq 5$  mice per group). **(C)** Image of AAV9-*hSyn*-iCre-p2a-KHC-  
969 CBD-injected P9 triangularis sterni muscle of a YFP reporter mouse immunostained for Caspr (green) and  
970  $\beta$ III-tubulin (white). KHC-CBD is overexpressed in iCre-induced recombined YFP reporter-positive axons  
971 (red). Schematic on the right depicts YFP-positive (red) and -negative motor units (gray), Caspr paranodes  
972 (green). **(D)** Quantification of Caspr-immunostaining on YFP-negative and -positive terminal axon branches  
973 at P9 ( $n \geq 5$  mice per group,  $n \geq 39$  axons per mouse). 'Din', competing axons; 'sin', winner axons. Data,  
974 mean + SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ , Mann-Whitney test. Outlier determined by Tukey  
975 test. Scale bar, 20  $\mu\text{m}$  in (C).

976

### 977 **Figure 6 | Nrg1 type III transgenic mice show premature myelination initiation.**

978 **(A, B)** P9 spinal cord of WT control (A) and *Thy1*-Nrg1 type III-HA (B) littermates. Sections stained for HA-  
979 tag (red) and neurotrace (cyan). Dashed boxes enlarged on the right, showing magnified single channel of  
980 neurotrace (cyan) and HA staining (red). **(C, D)** Confocal images of P9 triangularis sterni muscles from (C)

981 WT and (D) *Thy1-Nrg1* type III-HA littermates immunostained for  $\beta$ III-tubulin (white) and Caspr (green).  
982 Schematics below, motor neurons (gray), Caspr paranodes (green). Black arrowheads point to two axons  
983 leading to the same NMJ. **(E, F)** Quantification of the percentage of (E) doubly innervated NMJs and (F)  
984 Caspr-positive terminal branches in P7 and P9 WT vs. transgenic *Thy1-Nrg1* type III littermates. (E,  $n \geq 3$   
985 mice per genotype,  $\geq 99$  axons per animal; F,  $n \geq 3$  mice per genotype,  $\geq 40$  axons per animal). ‘Din’,  
986 competing axons; ‘sin’, winner axons. Data, mean + SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Mann-Whitney test.  
987 Scale bars, 10  $\mu$ m in (A–D).

988

989 **Figure 7 | Nrg1 type III is more concentrated on singly innervating terminal branches.**

990 **(A, B)** *Plp*-GFP x *Thy1-Nrg1* type III-HA mouse immunostained for neurofilament (NF, white) in P9  
991 triangularis sterni muscle. A stacked overview of competing (‘din’) vs. winner branches (‘sin’); dashed  
992 boxes enlarged in (B), showing magnified single optical sections of HA staining (red) with GFP labeled  
993 Schwann cells (green). **(C)** Quantification of HA staining on doubly vs. singly innervating branches in *Thy1-*  
994 *Nrg1* type III animals ( $n = 8$  mice per genotype,  $\geq 13$  axons per animal). **(D, E)** *Plp*-GFP x *Thy1-Nrg1* type III-  
995 HA mice immunostained for  $\beta$ III-tubulin (white) in P9 triangularis sterni muscle. A stacked overview (D) of  
996 competing (‘din’) vs. winner branches (‘sin’); dashed boxes enlarged in (E), showing magnified single optical  
997 sections of pERK staining (magenta) with GFP labeled Schwann cells (green). **(F)** Quantification of pERK  
998 staining around doubly vs. singly innervating branches in *Thy1-Nrg1* type III animals ( $n = 5$  mice per  
999 genotype,  $\geq 20$  axons per animal). **(G)** Quantification of pAKT immunostaining around doubly vs. singly  
1000 innervating branches in *Thy1-Nrg1* type III animals, normalized to singly innervating branches ( $\geq 20$  axons  
1001 per group in  $n = 5$  mice). **(H)** Quantification of HA signal in singly innervating axons in BTX injected  
1002 triangularis sterni muscle vs. uninjected control side ( $\geq 13$  axons per group in  $n = 6$  mice). **(I)** Quantification  
1003 of pERK signal in Schwann cells surrounding singly innervating axons in BTX injected triangularis sterni  
1004 muscle vs. uninjected control side ( $\geq 36$  axons per group in  $n = 5$  mice). **(J)** BTX intensity measured in wild-  
1005 type and *Thy1-Nrg1* type III transgenic animals (wild-type:  $698 \pm 67$  A.U., *Thy1-Nrg1* type III:  $747 \pm 43$  A.U.,

1006  $n \geq 16$  NMJ per animal,  $n \geq 5$  mice per group). **(K)** Area of BTX-stained endplate measured in wild-type and  
1007 *Thy1-Nrg1* type III transgenic animals (wild-type:  $195 \pm 13 \mu\text{m}^2$ , *Thy1-Nrg1* type III:  $203 \pm 22 \mu\text{m}^2$ ,  $n \geq 16$   
1008 NMJ per animal,  $n \geq 5$  mice per group). **(L)** Quantification of the proportions of NMJ morphology,  
1009 categorized into 'broken', 'holes' and 'plaque' ( $n \geq 5$  mice per group,  $\geq 14$  NMJ per animal). 'Din', competing  
1010 axons; 'sin', winner axons. Data, mean + SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , Mann-Whitney test. Scale bars, 10  
1011  $\mu\text{m}$  in (A) and (D); 5  $\mu\text{m}$  in (B) and (E).

## 1012 SUPPLEMENTARY FIGURE LEGENDS

### 1013 **Figure S1 | Characterization of *Thy1-Caspr-GFP* and *Thy1- $\beta$ 1-Nav-GFP* mice.**

1014 **(A)** Confocal image of P9 *Thy1-Caspr-GFP* (native GFP, green) intercostal axons ( $\beta$ III-tubulin, white)  
1015 immunostained for Caspr (red). Dashed boxes enlarged below show single channels. The percentage of  
1016 GFP-positive paranodes nodes was stable across development, suggesting consistent labeling of a neuronal  
1017 subset (P9-11:  $65 \pm 8$  % of all paranodal structures; 6 weeks:  $73 \pm 9$  %;  $P = 0.7$ , Mann-Whitney test;  $n = 4$   
1018 mice per age group,  $\geq 44$  nodes per animal). **(B)** *Triangularis sterni* muscle of a P9 *Thy1-Caspr-GFP* mouse  
1019 immunostained for Caspr (red) and axons ( $\beta$ III-tubulin, white). Dashed boxes enlarged below, showing  
1020 Caspr/GFP double-positive (i) and Caspr only-positive paranode (ii). Expression of the Caspr-GFP transgene  
1021 did not detectably influence the degree of double innervation (WT:  $9 \pm 1$  % vs. Caspr-GFP:  $12 \pm 2$  %;  $P =$   
1022  $0.4$ , Mann-Whitney test;  $n = 3$  mice per genotype,  $\geq 136$  axons per animal) or myelination on terminal axon  
1023 branches at P9 (winner branches - WT:  $32 \pm 2$  % vs. Caspr-GFP:  $35 \pm 8$  %; competing branches - WT:  $12 \pm$   
1024  $6$  % vs. Caspr-GFP:  $7 \pm 7$  %;  $P > 0.99$ , Mann-Whitney test;  $n = 3$  mice per genotype,  $\geq 31$  axons per animal).  
1025 **(C)** Image of P9 *Thy1- $\beta$ 1-Nav-GFP* (native GFP, green) intercostal axons ( $\beta$ III-tubulin, white) immunostained  
1026 for Nav (red). Dashed boxes enlarged below show single channels. All nodes identified by immunostaining  
1027 were also GFP-positive, indicating transgene expression in all motor neurons ( $100 \pm 0$  %;  $n = 3$  mice,  $\geq 40$   
1028 axons per animal). **(D)** *Triangularis sterni* muscle of a P9 *Thy1- $\beta$ 1-Nav-GFP* mouse immunostained for Nav

1029 (red) along terminal axon branches ( $\beta$ III-tubulin, white). Insets show enlarged Nav/GFP double-positive  
1030 nodes. Expression of the  $\beta$ 1-Nav-GFP transgene did not detectably influence the degree of double  
1031 innervation (WT:  $11 \pm 1$  % vs.  $\beta$ 1-Nav-GFP:  $14 \pm 2$  %;  $n = 3$  mice per genotype,  $\geq 102$  axons;  $P = 0.7$ , Mann-  
1032 Whitney test; axons per animal) or myelination on terminal axon branches at P9 (winner branches - WT:  
1033  $38 \pm 8$  % vs.  $\beta$ 1-Nav-GFP:  $30 \pm 4$  %; competing branches - WT:  $19 \pm 3$  % vs.  $\beta$ 1-Nav-GFP:  $11 \pm 6$  %;  $P > 0.4$ ,  
1034 Mann-Whitney test;  $n = 3$  mice per genotype,  $\geq 31$  axons per animal). 'Din', competing axons; 'sin', winner  
1035 axons. Scale bars,  $10 \mu\text{m}$  in (A–D) overview,  $2 \mu\text{m}$  in insets.

1036

1037 **Figure S2 | Innervation and myelination status correlate with axonal tubulin content and SC**  
1038 **length.**

1039 **(A)** Images of Schwann cells on singly innervating terminal branches in *Pfp*-GFP (green) mouse following  
1040 BTX injection on P7 vs. contralateral control side and post-hoc staining at P9 with  $\beta$ III-tubulin (white).  
1041 Schematics to the right depict measured terminal axon length (gray) and Schwann cell outline with cell  
1042 nuclei marked with asterisks. **(B–D)** Quantification of **(B)** Schwann cell length, **(C)** terminal branch length  
1043 and **(D)** Schwann cell number along singly innervating branches, showing no significant difference after  
1044 BTX treatment in P9 *Pfp*-GFP mice injected with BTX vs. control ( $\geq 10$  axons per animal in  $n = 5$  mice). **(E–F)**  
1045 Quantification of **(E)** axonal Schwann cell length (din:  $30 \pm 2 \mu\text{m}$ ; sin:  $24 \pm 1 \mu\text{m}$ ) and **(F)** terminal branch  
1046 length (din:  $50 \pm 4 \mu\text{m}$ ; sin:  $54 \pm 5 \mu\text{m}$ ;  $\geq 16$  axons per animal in  $n = 5$  mice). **(G)** Images of competing ('din')  
1047 and winner ('sin') terminal branches in P9 Thy1-YFP16 mice, without or with emerging Caspr paranodes  
1048 (green) and stained  $\beta$ III-tubulin (white). **(H)** Quantification of  $\beta$ III-tubulin intensity (x-fold normalized to  
1049 Thy1-YFP16; Caspr-  $n \geq 18$  axons per group in  $n = 3$  mice). 'Din', competing axons; 'sin', winner axons. Data,  
1050 mean + SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; Mann-Whitney test. Outlier determined by Tukey  
1051 test. Scale bar,  $10 \mu\text{m}$  in (A) and (G).

1052 **Figure S3 | AAV9 mediated spastin deletion promotes myelination on competing branches.**

1053 **(A)** Schematic of experimental design. AAV9-*CMV*-iCre was injected at P2 into the 3<sup>rd</sup> ventricle of spastin<sup>fl/fl</sup>  
1054 x TdTomato reporter mice. Muscles were analyzed at P9. **(B)** Image of P9 muscle immunostained for Caspr  
1055 (green) and  $\beta$ III-tubulin (white). iCre-mediated deletion resulted in TdTomato-positive axons (red),  
1056 presumed to lack spastin. Schematic on the right depicts TdTomato-positive (red) and -negative motor  
1057 units (gray), Caspr paranodes (green). Black arrowheads point to competing axons leading to the same  
1058 NMJ. **(C)** Quantification of Caspr-immunostaining on TdTomato-negative and -positive terminal branches  
1059 at P9 ( $n \geq 3$  mice per group,  $n \geq 15$  axons per mouse). **(D)** Quantification of axon diameter of TdTomato-  
1060 negative and -positive terminal branches at P9 ( $n \geq 10$  axons per group,  $n = 5$  mice). **(E)** Schematic of  
1061 experimental design. AAV9-*CMV*-iCre was injected at P2 into the 3<sup>rd</sup> ventricle of Nrg1 type III<sup>fl/fl</sup> x TdTomato  
1062 reporter mice. Muscles were analyzed at P9. **(F)** Image of P9 muscle immunostained for Caspr (green) and  
1063  $\beta$ III-tubulin (white). iCre-mediated deletion resulted in TdTomato-positive axons (red), presumed to lack  
1064 Nrg1. Schematic on the right depicts TdTomato-positive (red) and -negative motor units (gray), Caspr  
1065 paranodes (green). Black arrowheads point to two axons leading to the same NMJ. **(G)** Quantification of  
1066 doubly innervated NMJs on TdTomato-negative and -positive terminal branches at P9 ( $n = 4$  mice per group,  
1067  $\geq 97$  axons per animal). **(H)** Quantification of Caspr-immunostaining on TdTomato-negative and -positive  
1068 terminal branches at P9 ( $n = 4$  mice per group,  $\geq 29$  axons per animal). ‘Din’, competing axons; ‘sin’, winner  
1069 axons. Data, mean + SEM. \*,  $P < 0.05$ , Mann-Whitney test. Outlier determined by Tukey test. Scale bar, 10  
1070  $\mu$ m in (B) and (F).

1071

1072 **Figure S4 | Microtubule-dependent axonal transport affects myelination onset.**

1073 **(A–D)** Whole-mount immunohistochemical staining against  $\alpha$ -tub (white) to label axons in Tg(mbp:RFP)  
1074 (magenta) transgenic zebrafish larvae injected with cntn1b:GFP as control (A, B) and cntn1b:GFP-KHC-CBD  
1075 (C, D). Dashed boxes in (A, C) are enlarged in (B, D) showing mbp:RFP only. **(E)** Example of an individual

1076 cntn1b:GFP-KHC-CBD labelled motor neuron (yellow) and its myelination (magenta). Solid arrow heads  
1077 point to ends of myelin sheaths; empty arrow head points to extend of myelination along KHC-CBD  
1078 expressing axons compared to control axons in the adjacent somite (unlabeled). **(F)** Length of spinal motor  
1079 axons, measured between the branching-off point at the spinal cord to the axon tip (n = 7 zebrafish per  
1080 group, n ≥ 29 axons per animal). **(G)** Progress of myelination expressed as percentage of mbp:RFP-positive  
1081 axon length (n = 7 zebrafish per group, n ≥ 29 axons per animal). Data, mean + SEM. \*\*\*, P < 0.001, Mann-  
1082 Whitney test. Scale bar, 50 μm in (A–E).



























