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LETTER TO THE EDITOR

Developmental pruning of early-stage myelin segments during CNS myelination *in vivo*

Dear Editor,

Axon-wrapping myelin sheath is essential for proper functioning of the nervous system. Myelin is formed from membranous sheets that are elaborated by oligodendrocytes (OLs) in the central nervous system (CNS). Although much is known about the development and migration of OL and its progenitor cell *in vitro* and *in vivo* [1-3], how CNS myelin is dynamically formed through OL processes is still unclear [1, 3]. To examine the process of CNS myelination *in vivo*, we performed long-term time-lapse confocal imaging of the same OLs in the spinal cord of intact zebrafish larvae during 2-7 days post-fertilization (dpf) with intervals of 2-12 h. Individual OLs were mosaically labeled by membrane-tethered red fluorescent protein (mRFP) under the control of zebrafish *Sox10* regulatory DNA [2]. We find that, besides extension of some early-stage axon-wrapping myelin segments, 28% segments undergo pruning in an axon diameter-correlated manner during early development. Downregulating neuregulin-ErbB signaling impairs the pruning but not extension. Thus, our study reveals early-stage myelin pruning as a novel process during myelination.

First, we found that, in zebrafish larvae, OLs initially emanated numerous processes (Figure 1A$_1$), and then synchronously formed multiple early-stage myelin segments at 80.6 ± 9.7 h post-fertilization (hpf; Figure 1A$_2$ and 1B). To simultaneously visualize axons, transgenic *Tg(HuC:GFP)* zebrafish, in which some axons in the spinal cord express green fluorescent protein (GFP), were used (Supplementary information, Figure S1). To quantify the myelination process, we measured the total number of early-stage myelin segments in individual OLs and the length of each segment at each time point. Interestingly, the total number of segments gradually decreased from 12.5 ± 1.1 at the initiation time point to 8.3 ± 0.8 after 4 days (*P* < 0.01; Figure 1A$_2$-1A$_6$ and C). This decrease was mainly due to the fact that, after the initiation of myelin segments, although many of them continued to extend in their length ("extending segment"), 28.6

± 3.4% of these early-stage myelin segments underwent retraction and final pruning in 25 out of 27 OLs examined ("pruning segment"; Figure 1D and 1E). The mean lifespan of pruning segments was about 35.8 ± 2.2 h, and positively correlated with their initial length (*P* < 0.0001; Supplementary information, Figure S2). These results raise the possibility that, besides myelin extension, myelin pruning may be another important process during myelination.

Next, we found that this early-stage myelin pruning preferred to occur at small but not large axons. In the spinal cord of zebrafish, the Mauthner ("M") cell axon, the largest one in the animal, is the first to be myelinated [4]. Some OLs initially formed myelin segments on both M-axons and other small axons (Figure 1F and Supplementary information, Figure S3). In those OLs, early-stage myelin segments on M-axons never underwent pruning (0 out of 15; "M-axon's myelin segment"; Figure 1F and 1G, and Supplementary information, Figure S3), whereas segments on small axons by the same OL ("small axon's myelin segment of M-axon-wrapping OL") exhibited faster pruning with a higher probability in comparison with the segments of OLs which did not wrap M-axons ("small axon's myelin segment of non-M-axon-wrapping OL"; Figure 1G and 1H).

The membrane-bound neuregulin1 (NRG1) is an axonal ligand for glial ErbB receptors, and NRG1-ErbB signaling is required for myelination in both the peripheral and central nervous systems [5-8]. To examine whether this signaling pathway has a role in the early-stage myelin pruning, we first used two previously characterized morpholino oligomers (MOs) to knock down the expression of NRG1 in zebrafish larvae. In NRG1 knockdown larvae (morphants), the number of initial myelin segments was not significantly different from that in wild-type larvae (Figure 1I and Supplementary information, Figure S4A and S4B). However, the probability of early-stage myelin segment pruning in *NRG1* morphants was markedly decreased (*NRG1* morphant *vs* Control: 11.9 ± 2.0% *vs* 28.6 ± 3.4%, *P* < 0.01; Figure 1J), and the process of residual pruning events was also slowed down
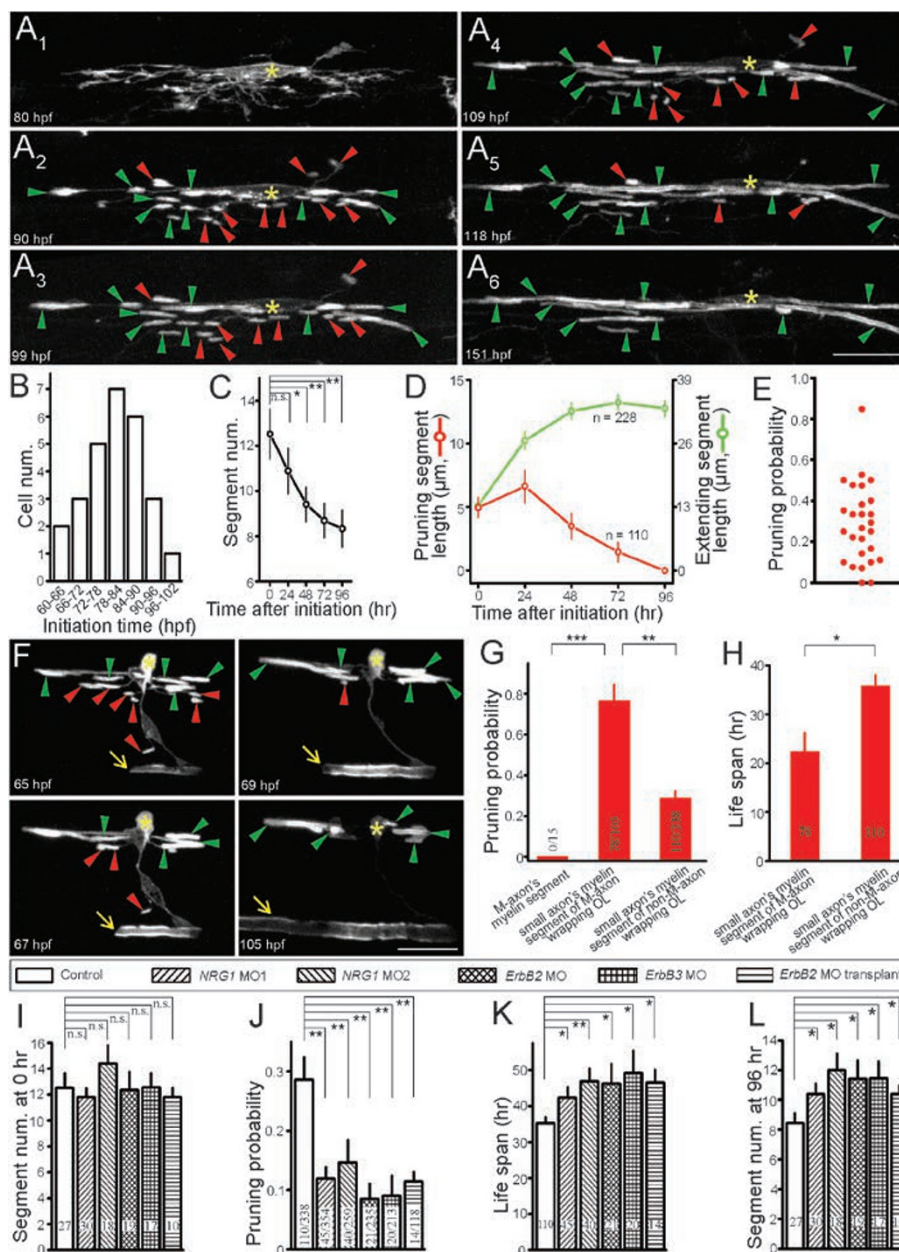
Figure 1 Pruning of early-stage myelin segments during oligodendrocyte myelination. **(A₁-A₆)** Time-lapse images of an oligodendrocyte (OL) in the spinal cord of a zebrafish larva. Lateral views with dorsal up and anterior left. Asterisk, OL soma; Green arrowhead, extending myelin segment; Red arrowhead, pruning myelin segments; hpf, hours post-fertilization. Scale: 20 μm. **(B)** Initiation time of myelination. Data were obtained from 27 OLs in 21 animals. **(C)** Time course showing change in the number of myelin segment after initiation. Same data set with **B** was used. hr, hour. **(D)** Time course showing changes in the mean length of pruning (red) and extending (green) segments after initiation. The numbers indicate the numbers of myelin segments examined in 27 OLs. **(E)** Pruning probability of early-stage myelin segments in individual OLs (*n* = 27). Same data set with **B** was used. The pruning probability was calculated by dividing the number of pruning segments after 96 h with the total segment number at the initiation time. **(F-H)** Axon diameter-dependency of myelin segment pruning. **(F)** Time-lapse images of an OL, one process of which wrapped Mauthner cell axon (arrow). Asterisk, OL soma; Green arrowhead, extending myelin segment; Red arrowhead, pruning myelin segments. Scale, 20 μm. **(G)** Pruning probability of different types of myelin segments. Number/number, pruning segment number/total segment number. **(H)** Lifespan of pruning myelin segments of M-axon- and non-M-axon-wrapping OLs. The numbers on bars represent the numbers of pruning segments examined. **(I-L)** NRG1-ErbB signaling is required for myelin segment pruning. The data obtained from OLs without contacting Mauthner cell axons were analyzed. **(I)** Mean number of total myelin segments per OLs at the myelination initiation timepoint. The numbers on bars represent the numbers of OLs examined. **(J)** Pruning probability of myelin segments. Number/number, pruning segment number/total segment number. **(K)** Life span of pruning myelin segments. **(L)** Mean number of total myelin segments per OLs 96 h after myelination initiation. n.s., no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (unpaired Student's *t*-test and one-way ANOVA). Error bars represent mean ± SEM.

($P < 0.05$; Figure 1K), leading to an increase in the total number of myelin segments examined 96 h later ($P < 0.05$; Figure 1L). Meanwhile, the growth rate of extending myelin segments was not significantly affected (Supplementary information, Figure S4E). As a result, the total length of segments in individual OLs was increased in NRG1 morphants ($P < 0.05$; Supplementary information, Figure S4F). Similar results were observed when the expression of ErbB2 or ErbB3 was downregulated (Figure 1I-1L and Supplementary information, Figure S4). These results suggest that NRG1-ErbB signaling is important for the early-stage myelin segment pruning.

To examine whether ErbB expressed in oligodendrocytes is required, we transplanted some cells at the blastula stage from donor transgenic *Tg(Sox10:mRFP)* embryos, which were injected with *ErbB2* morpholino, into wild-type host embryos. Thus, in chimera embryos, RFP-positive oligodendrocytes were originated from *ErbB2* morphants and carried deficiency in ErbB2. We found that the RFP-positive oligodendrocytes in chimeras initially formed numerous early-stage myelin segments with a number comparable to that under normal condition ("*ErbB2* MO transplant", Figure 1I). However, only few of those segments underwent pruning during development, similar to what we observed in *ErbB2* morphants (Figure 1J and Supplementary information, Figure S5). This indicates that ErbB2 in oligodendrocytes is required.

In the nervous system, pruning of neural processes and synapses is a common aspect during development, and ensures the establishment of proper neural connections [9]. Here, we find that early-formed axon-wrapping myelin segments also undergo extensive pruning, which may accompany axon pruning during development. Furthermore, segments wrapping larger axons will win and ones wrapping smaller axons will lose, raising the possibility that myelin pruning exhibits a competitive property. This may explain why large axons recruit glial wrapping early and receive more myelin layers than small axons [1, 4, 10]. During the pruning process, NRG-ErbB signaling may serve as a competitive signal at extending myelin segments or a pruning signal at pruning segments. It is of interest to discriminate these possibilities in the future.

Peng Liu[1], Jiu-lin Du[2], Cheng He[1]

[1]Department of Neurobiology and Key Laboratory of Molecular Neurobiology of Ministry of Education, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China; [2]Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China
Correspondence: Jiu-lin Du[a], Cheng He[b]
[a]Tel: +86-21-54921825; Fax: +86-21-54921735
E-mail: forestdu@ion.ac.cn
[b]Tel: +86-21-65515200; Fax: +86-21-65492132
E-mail: chenghe@smmu.edu.cn

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)