Individual axons regulate the myelinating potential of single oligodendrocytes in vivo

Rafael G. Almeida1,2,*, Tim Czopka1,2,3,*, Charles ffrench-Constant2,3 and David A. Lyons1,2,†

SUMMARY
The majority of axons in the central nervous system (CNS) are eventually myelinated by oligodendrocytes, but whether the timing and extent of myelination in vivo reflect intrinsic properties of oligodendrocytes, or are regulated by axons, remains undetermined. Here, we use zebrafish to study CNS myelination at single-cell resolution in vivo. We show that the large caliber Mauthner axon is the first to be myelinated (shortly before axons of smaller caliber) and that the presence of supernumerary large caliber Mauthner axons can profoundly affect myelination by single oligodendrocytes. Oligodendrocytes that typically myelinate just one Mauthner axon in wild type can myelinate multiple supernumerary Mauthner axons. Furthermore, oligodendrocytes that exclusively myelinate numerous smaller caliber axons in wild type can readily myelinate small caliber axons in addition to the much larger caliber supernumerary Mauthner axons. These data indicate that single oligodendrocytes can myelinate diverse axons and that their myelinating potential is actively regulated by individual axons.

KEY WORDS: CNS Myelination, In vivo imaging, Oligodendrocyte, Zebrafish

INTRODUCTION
The vast majority of axons in the vertebrate central nervous system are eventually myelinated by oligodendrocytes (Hildebrand et al., 1993), which facilitates rapid saltatory conduction along their length (Bakiri et al., 2011; Waxman and Swadlow, 1977), ensures their long-term viability (Nave, 2010) and contributes to functional regulation of nervous system plasticity (Fields, 2010). Although we know much about the early development of oligodendrocytes (Li et al., 2009; Richardson et al., 2006) and that oligodendrocytes can differentiate and express myelin proteins in the absence of axons in vitro (Barres et al., 1994; Knapp et al., 1987; Nawaz et al., 2009; Temple and Raff, 1986), the key question of whether the timing and extent of myelination in vivo represents intrinsic properties of oligodendrocytes or is regulated by the axons remains unanswered. Although it has been known for nearly 100 years that oligodendrocyte morphology correlates with the caliber of axons that they myelinate, whereby oligodendrocytes usually associate with either a small number of large caliber axons or a larger number of smaller axons (Bunge, 1968; Butt and Berry, 2000; Del Rio-Hortega, 1921; Del Rio-Hortega, 1928; Remahl and Hildebrand, 1990), a causal role for axons in establishing these relationships, although predicted (Ueda et al., 1999), remains to be formally demonstrated.

Here, we use zebrafish, which have recently become established as a powerful model for the study of glial cells and myelinated axons (Brosamle and Halpern, 2002; Buckley et al., 2010; Kazakova et al., 2006; Kirby et al., 2006; Li et al., 2007; Lyons et al., 2009; Monk et al., 2009; Pogoda et al., 2006; Takada and Appel, 2010), to study the role that axons play in regulating CNS myelination in vivo. As is the case in mammals, we show that single oligodendrocytes in zebrafish myelinate either a small number of large caliber axons or a larger number of smaller axons. We identify the very first axon myelinated in the zebrafish CNS as the very large caliber Mauthner axon, and show that increasing the number of Mauthner axons in vivo dramatically regulates myelination by single oligodendrocytes. In the presence of supernumerary Mauthner axons we observe oligodendrocytes that usually myelinate just one Mauthner axon myelinating many Mauthner axons. In addition, we observed oligodendrocytes that usually myelinate only smaller caliber axons readily myelinating both large caliber Mauthner axons as well as axons of much smaller caliber, which happens very rarely in wild types. These results show that oligodendrocytes can myelinate axons of very different size and that individual axons can regulate the myelinating potential and, thus, the morphology of single oligodendrocytes in vivo.

MATERIALS AND METHODS

Zebrafish
We used the following standard zebrafish strains and lines: AB, Golden and Tg(sox10(7.2):mRFP) (Kirby et al., 2006). For this study, we also generated Tg(mbp:EGFP-CAAX) and Tg(mbp:EGFP). All animals were maintained in accordance with UK Home Office guidelines.

Plasmid construction
A 2 kb fragment of mbp regulatory sequence (Jung et al., 2010) was amplified from genomic DNA of the AB strain using the following primers that contain att recombination sites (underlined): attB4_mbpF, GGGGACTGCTTTTTTGTACAAA-CTTGCAACTTTGTATAGAAAAGTTG; attB1R_mbpR, GGGGACTGCTTTTTTGTACAAA-CTTGCAACTTTGTATAGAAAAGTTG. Purified PCR products were recombined with pDONRP4-P1R (Invitrogen) using BP clonase (Invitrogen) to generate the 5′ element clone p5E mbp. Tol2 transgenesis constructs were generated by recombination of the abovementioned and other donor clones, all of which are components of the Tol2kit (Kwan et al., 2007), with pDEST_Tol2_CG2 using LR clonase II Plus (Invitrogen).

1Centre for Neuroregeneration, University of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, UK. 2Centre for Multiple Sclerosis Research, University of Edinburgh, 47-49 Little France Crescent, Edinburgh EH16 4SB, UK. 3MRC Centre for Regenerative Medicine, University of Edinburgh, 47-49 Little France Crescent, Edinburgh EH16 4SB, UK.
*These authors contributed equally to this work
†Author for correspondence (david.lyons@ed.ac.uk)

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DNA injections and generation of transgenic zebrafish
Fertilised eggs were co-injected with 1 nl of a solution containing 10 ng/µL plasmid DNA and 25 ng/µL tol2 transposase mRNA. Injected fish were analysed as mosaics or grown to adulthood to raise stable transgenic lines.

\textit{notch1a} morpholino injection

We injected zebrafish embryos with 500 pg of a morpholino directed against a \textit{notch1a}-specific splice junction (Ma and Jiang, 2007) to temporarily abrogate embryonic \textit{notch1a} expression. We carried out RT-PCR to assay \textit{notch1a} mRNA expression levels using the primers 5'-CTTCTGCACCTTTCTGGAGATTTAAAGAAG-3' (Ma and Jiang, 2007) and 5'-CACAGCCTGACCTGTAAGACG-3'.

\textit{hoxb1} mRNA injection

We injected zebrafish embryos with 50 pg of \textit{hoxb1} mRNA. The majority of \textit{hoxb1} mRNA-injected animals were morphologically normal and typically had two Mauthner axons on one side of the CNS and one on the contralateral side. A subset of \textit{hoxb1} mRNA-injected animals had morphological abnormalities as have been previously described (Hale et al., 2004), and were therefore excluded from further study.

Immunohistochemistry

Whole-mount antibody labelling was carried out using standard protocols. Anti-3A10 (Developmental Studies Hybridoma Bank) was used at a concentration of 1:200.

Confocal imaging

Embryonic and larval zebrafish were embedded in 1.5% low melting point agarose (Sigma) in embryo medium with tricaine. Imaging was performed at a Zeiss LSM710 confocal microscope. Quantification of cell and myelin sheath number was carried out on confocal z-stacks using unbiased stereological methods.

Transmission electron microscopy

Preparation of tissue was as carried out as described previously (Lyons et al., 2008). Microwave stimulation during tissue processing was carried out using a Panasonic microwave with ‘Inverter’ technology. Images were taken with a Phillips CM120 Biotwin. Data analyses were carried out using Adobe Photoshop and ImageJ.

RESULTS

CNS myelination proceeds according to stereotyped gradients in vivo

To define CNS myelination in vivo at single cell resolution, we generated transgenic constructs and stable transgenic lines to drive gene expression in myelinating glial cells of the zebrafish (Fig. 1A; see Fig. S1 in the supplementary material). Time-course analyses of fluorescent reporter lines generated using previously identified regulatory sequences of the myelin basic protein (\textit{mbp}) gene (Jung et al., 2010) showed that the very first axon to be myelinated in the CNS is that of the large Mauthner neuron (Fig. 1B). The Mauthner cell is an individually identifiable reticulospinal neuron; each fish has two Mauthner neurons, one on each side of the midline of rhombomere 4 (Kimmel et al., 1982) and each Mauthner neuron projects a very large caliber axon along a stereotyped path in the ventral spinal cord (Jontes et al., 2000; Kimmel et al., 1982). \textit{mbp}:EGFP-CAAX (which drives a membrane-tethered variant of EGFP) was first detected along proximal (anterior) parts of the Mauthner axon from about 60 hours post-fertilisation (hpf) and proceeded progressively towards more distal (posterior) parts of the axon over time (Fig. 1B, C). Myelination of axons in more dorsal regions of the spinal cord occurred soon after the appearance of myelination of the ventral Mauthner axon (Fig. 1C). Myelination of these more dorsally located axons also occurred in an anterior to posterior gradient (data not shown). These observations show that myelination occurs according to stereotyped anterior-posterior and ventral-dorsal gradients at the level of axonal tracts and individual axons.

Individual myelinating oligodendrocytes have diverse morphologies in vivo

In order to visualise individual myelinating oligodendrocytes in vivo we injected wild-type embryos with plasmid DNA encoding \textit{mbp}:EGFP or \textit{mbp}:EGFP-CAAX and imaged them between 3 and 9 days post-fertilisation (dpf). We observed that the oligodendrocytes that associate with the very large Mauthner axon typically associate only with this axon (Fig. 2A). The vast majority of such cells (103/133) associated with just one Mauthner axon, whereas the remaining cells associated with the Mauthner axon on both sides of the embryonic midline (Fig. 2A). We also found that a very small proportion of wild-type oligodendrocytes (5%, 11/204) were capable of myelinating the Mauthner axon as well as axons of much smaller caliber (Fig. 2B). The position of oligodendrocytes that associate with the Mauthner axons was very stereotyped: the cell bodies always resided in the ventral spinal cord, almost always ventral to the Mauthner axon itself (Fig. 1B, C; Fig. 2A, B).

As expected, the vast majority of oligodendrocytes in the spinal cord extended multiple myelinating processes that associated with numerous axons of relatively small caliber compared with the Mauthner axons. These cells exhibited striking morphological diversity with respect to the number and length of their individual
myelin sheaths (Fig. 2C-E). At 4 dpf, we found that the majority of such cells (14 of 22) had 11-14 myelinating processes, although process number varied between 4 and 22 (Fig. 2D). Also, the number of such cells (14 of 22) had 11-14 myelinating processes, although process number varied between 4 and 22. Another method to generate extra Mauthner axons is by duplicating rhombomere 4 to 6, which has previously been shown to be capable of generating animals with ectopic Mauthner neurons by duplicating rhombomere 4 identity in the hindbrain (Hale et al., 2004).

Examination of notch1a morphants and embryos injected with hoxb1 mRNAs between 3 and 9 dpf showed that normal and supernumerary Mauthner axons were covered by glial membrane and robustly myelinated (Fig. 3; Fig. 4; data not shown). The Mauthner axon, like many other axons in situ, is not circular in cross-sectional profile (Fig. 4). We therefore quantified the circumference(s) of Mauthner axon(s) in our analyses, because this variable (in combination with axon length) reflects the axonal surface available for potential interactions with oligodendrocytes. We saw that the summed circumference(s) of all Mauthner axon(s) per hemi-spinal cord increased almost linearly with the number of Mauthner axons present (Fig. 4D). In control animals that have one Mauthner axon on each side of the spinal cord, the average circumference per axon was 13.5±0.8 μm, whereas animals with two or more Mauthner axons on one side of the spinal cord, the combined circumference was approximately doubled (26±3.5 μm, P<0.0001) and those with three on each side nearly tripled (35±5 μm, P<0.0001). In animals with even more Mauthner axons, their total circumference increased even further.

In order to test whether myelination of extra Mauthner axons occurred at the expense of other axons, we counted the total number of myelinated axons (excluding the Mauthner axons) in control and experimental ventral spinal cords (Fig. 4E). Control
animals with one Mauthner axon on each side of the spinal cord had an average of 23 additional myelinated axons (±4, n=10). In animals with two Mauthner axons on each side of the spinal cord there were 22 additional axons myelinated (±4, n=10, P=0.58) as was also the case in animals with three Mauthner axons (n=3, P=0.71). These data show that the presence of even a threefold increase in Mauthner axon circumference does not affect the number of other axons myelinated in the ventral spinal cord. There was, however, a slight decrease in the number of myelinated axons when there were four or more Mauthner axons present in one side of the ventral spinal cord (15±3, n=4, P=0.0038).

Together, our results show that in two genetically independent manipulations oligodendrocytes can myelinate extra large caliber axons that are not present in their normal wild-type environment and can do so without affecting myelination of other axons. These observations beg the question as to how oligodendrocytes respond to the presence of extra axons and their consequence additional axonal surface area.

**Supernumerary Mauthner axons do not regulate myelinating oligodendrocyte number**

In order to test whether additional Mauthner axons regulate early stages of oligodendrocyte development, we examined the number and distribution of oligodendrocytes in *notch1* morphants and animals with *hoxb1* mRNA overexpression. We found no significant differences in oligodendrocyte number in the spinal cords of animals with supernumerary Mauthner axons relative to controls over a 425 µm stretch of the spinal cord centred at the mid-trunk level: control animals had an average of 66 mbp:EGFP-expressing oligodendrocytes (±6, n=6) at 5 dpf; *notch1* morphants an average of 67 (±5, n=10, P=0.72); and *hoxb1* mRNA-injected animals an average of 63 (±8, n=9, P=0.45) (Fig. 5). Wild-type and supernumerary Mauthner axons are localised to the ventral spinal cord, as are the oligodendrocytes that typically myelinate them (Fig. 1), and given that the Mauthner axon is the first myelinated, we wanted to test the possibility that the distribution of oligodendrocytes within the spinal cord might be affected by the presence of supernumerary Mauthner axons in the ventral spinal cord. We saw, however, that the number of oligodendrocyte cell bodies located specifically in the dorsal spinal cord was almost identical in wild type as in animals with extra Mauthner axons (control: 25±3, n=6; *notch1* morphants: 23±4, n=10, P=0.31; *hoxb1* mRNA-injected animals: 21±7, n=9, P=0.21) (Fig. 5D).

**Supernumerary Mauthner axons regulate the morphology of single oligodendrocytes**

In order to elucidate precisely how supernumerary Mauthner axons regulate single myelinating oligodendrocytes, we injected plasmid DNA encoding mbp:EGFP or mbp:EGFP-CAAX into *notch1* morphants and into embryos injected with *hoxb1* mRNA. Whereas ~80% of the oligodendrocytes that myelinated the Mauthner axon in wild type associated with only one Mauthner axon, we found no such oligodendrocytes in *notch1* morphants. Instead, we saw that all oligodendrocytes associated with at least two Mauthner axons (n=22) and that the majority of these associated with at least three Mauthner axons (15/22) (Fig. 6). In one case, we saw an oligodendrocyte that associated with seven Mauthner axons (Fig. 6C). In animals injected with *hoxb1* mRNA we also found that the vast majority of oligodendrocytes associated with more than one Mauthner axon (16/21) (Fig. 6). In wild-type animals, the average total length of myelin sheaths along Mauthner axon(s) made by single oligodendrocytes was 150±47 µm, in *notch1* morphants was 256±57 µm (P<0.0001) and in *hoxb1* mRNA-injected animals was 187±85 µm (P=0.05) at 5 dpf. By 9 dpf, the average total lengths were 192±53 µm in wild type, 419±95 µm (P<0.0001) in *notch1* morphants and 249±107 µm (P=0.09) in *hoxb1* mRNA-injected animals. Together with the fact that the total circumference of supernumerary Mauthner axons far exceeds the circumference...
of individual wild-type Mauthner axons (Fig. 4D), it is clear that individual oligodendrocytes in animals with supernumerary Mauthner axons myelinate a far greater axonal surface area than do wild-type oligodendrocytes.

In addition to identifying oligodendrocytes that myelinated a greater number of Mauthner axons than normal, we observed that oligodendrocytes in the ventral spinal cord can myelinate supernumerary Mauthner axons as well as axons of relatively smaller caliber (data not shown). Remarkably, we also saw oligodendrocytes located in dorsal regions of the spinal cord, which typically only ever myelinate small caliber axons, extend processes to the ventral spinal cord to associate with supernumerary Mauthner axons, and in many cases also myelinate much smaller caliber axons (Fig. 7). This was never observed in wild-type animals but occurred readily in both notch1 morphants and embryos injected with hoxb1 mRNA, and at both 5 dpf and 9 dpf, which shows that individual oligodendrocytes can readily myelinate axons of very different size and that axons profoundly affect the myelination capacity of single oligodendrocytes.

**DISCUSSION**

Here, we use zebrafish to characterise and manipulate CNS myelination in vivo. Zebrafish are ideally suited to cellular analyses at high resolution owing to their small size, optical transparency, rapid early development, accessibility and relative simplicity compared with mammals, in which the vast majority of studies of myelinated axons have been carried out. By analysing the morphology of single zebrafish oligodendrocytes in vivo using fluorescent transgenes we recapitulate key observations made in mammals nearly 100 years ago by Pio Del Rio-Hortega (Del Rio-Hortega, 1921; Del Rio-Hortega, 1928), which showed that oligodendrocytes typically myelinate either a...
small number of large caliber axons or a larger number of smaller caliber axons in situ. By virtue of being able to visualise myelination from its very onset at single cell resolution in vivo, we identify the large caliber Mauthner axon as the first to be myelinated in the zebrafish CNS, and show that this individual axon is myelinated along a proximal to distal gradient. We make use of two independent genetic manipulations to generate animals that have extra large caliber Mauthner axons and show that these axons can dramatically affect oligodendrocyte behaviour. Our manipulation of large caliber axon number shows that individual oligodendrocytes can readily myelinate axons of vastly different caliber in vivo and that myelination cues are, therefore, independent of axon subtype, a result that was predicted by previous transplantation studies in which oligodendrocytes derived from the optic nerve (which contains primarily small caliber axons) were capable of myelinating axons in the spinal cord (which contains axons of different sizes) (Fanarraga et al., 1998).

Our data that the presence of supernumerary large caliber Mauthner axons does not affect oligodendrocyte number are, at first glance, at odds with a previous study which showed that an increase in optic nerve axon number caused a concomitant increase in oligodendrocyte number (Burne et al., 1996). Indeed, although recent evidence has suggested that cell-cell interactions between neighbouring oligodendrocytes might contribute to regulation of their distribution and number (Kirby et al., 2006), there is extensive evidence from previous studies that axons can regulate oligodendrocyte number by affecting their proliferation and survival (Barres and Raff, 1999). However, it is important to point out that in the study of Burne et al. total axonal number was increased nearly twofold, whereas our manipulation simply increases the number of very large caliber axons during the onset of myelination and has a negligible effect on overall axon number. We do not, therefore, rule out a general role for axons in regulating oligodendrocyte number, but show instead that individual axons can dramatically regulate the myelination potential of single oligodendrocytes.

Although our data indicate that individual axons regulate CNS myelination in vivo, we do not know the identity of the causative axonal signal(s). Previous molecular characterisation has suggested that removal of inhibitory axonal cues is a pre-requisite for CNS myelination. Axons initially express high levels of polysialylated neural cell adhesion molecule (PSA-NCAM) on their surfaces, and myelination commences only when PSA-NCAM levels are reduced (Charles et al., 2000; Fewou et al., 2007; Jakovcevski et
Axons regulate OL morphology

RESEARCH ARTICLE

al., 2007; Keirstead et al., 1999). Analyses of the transmembrane protein Ling01 (Lee et al., 2007; Mi et al., 2005) also suggest that it too might function on axons to inhibit myelination. In the peripheral nervous system (PNS) there is strong evidence that axonal Neuregulin 1 type III serves as an instructive cue for myelination (Michailov et al., 2004; Tavveggi et al., 2005). Although Neuregulin 1 type III is not required for CNS myelination in vivo, it can stimulate hypermyelination of CNS axons in vivo (Brinkmann et al., 2008), which indicates that oligodendrocytes can respond to instructive axonal cues. It will be interesting to see whether future studies will identify instructive axonal signals required for CNS myelination.

Our data that single oligodendrocytes faced with supernumerary large caliber Mauthner axons can myelinate a larger axonal surface area than they normally do during development prompts the question of whether there is a maximum amount of axonal surface that any single oligodendrocyte can myelinate. If single oligodendrocytes myelinate close to their maximum capacity, e.g. by adulthood, then unless every oligodendrocyte is replaced following demyelination after injury or disease, incomplete repair might be inevitable. Our demonstration that axon size can regulate oligodendrocyte morphology is also relevant to the remyelination of axons. Given the significant cross-sectional growth of myelinated axons that takes place after the onset of myelination, it is likely that the caliber of any axon demyelinated long after development will be significantly larger than when it was first myelinated. This discrepancy in target caliber might mean that the morphology of remyelinating oligodendrocytes will be different than during development. It is possible that during remyelination more individual oligodendrocytes make fewer myelin sheaths on such larger caliber axons (Blakemore, 1974). If this is indeed the case, remyelination might necessitate the employment of a greater number of oligodendrocytes than are required to myelinate the same complement of axons during development. It is, however, also possible that oligodendrocytes faced with such relatively large caliber axons during remyelination do not regulate their morphology dramatically, but rather extend less myelin around individual axons. This possibility could explain the fact that remyelinated axon profiles are often surrounded by thin myelin (Blakemore, 1974). Future studies of demyelination and remyelination may benefit from high-resolution analyses that can assess precisely how much axonal surface is myelinated or how much myelin is made by individual oligodendrocytes.

In summary, our study shows that individual axons can profoundly regulate the myelinating potential of single oligodendrocytes, which has implications for the formation and regulation of myelinated axons throughout life and for mechanisms that may need to be considered during their repair.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


