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Title:
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Date:
2018-10-01

Citation:
Blades, F., Aprico, A., Akkermann, R., Ellis, S., Binder, M. D. & Kilpatrick, T. J. (2018). The TAM receptor TYRO3 is a critical regulator of myelin thickness in the central nervous system. GLIA, 66 (10), pp.2209-2220. https://doi.org/10.1002/glia.23481.

Persistent Link:
https://hdl.handle.net/11343/284462

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TITLE:

THE TAM RECEPTOR TYRO3 IS A CRITICAL REGULATOR OF MYELIN THICKNESS IN THE CENTRAL NERVOUS SYSTEM

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RUNNING TITLE:

TYRO3 REGULATES MYELIN THICKNESS

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NUMBER OF WORDS: 220 (Abstract), 620 (Introduction), 742 (Materials and Methods), 1463 (Results), 1583 (Discussion), 126 (Acknowledgements), 1098 (Figure Legends), 1059 (Bibliography)

NUMBER OF FIGURES AND TABLES: 6 Figures, 0 Supplementary figures, 0 Tables

TOTAL NUMBER OF WORDS: 6385

MAIN POINTS:

Myelin is thinner in the absence of Tyro3

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/glia.23481

Loss of Tyro3 increases the rate of cuprizone-induced demyelination

The absence of Tyro3 reduces efficiency of myelin repair following demyelination

KEYWORDS:

Oligodendrocyte, axon ensheathment, myelin wrap, remyelination, demyelination

ABSTRACT

Multiple sclerosis (MS) is an autoimmune, demyelinating disease of the central nervous system (CNS). Major deficits arise in MS patients due to an inability to repair damaged myelin sheaths following CNS insult, resulting in prolonged axonal exposure and neurodegeneration. The TAM receptors (Tyro3, Axl and Mertk) have been implicated in MS susceptibility, demyelination and remyelination. Previously, we have shown that Tyro3 regulates developmental myelination and myelin thickness within the optic nerve and rostral region of the corpus callosum (CC) of unchallenged adult mice. In this study we have verified and extended our previous findings via a comprehensive analysis of axonal ensheathment and myelin thickness in the CC of unchallenged mice, as well as following demyelination and during myelin repair. We have found that the loss of the Tyro3 receptor correlates with significantly thinner myelin sheaths in both unchallenged mice and during remyelination, particularly in larger caliber axons. The hypomyelinated phenotype observed in the absence of Tyro3 occurs independently of any influence upon OPC maturation, or density of oligodendrocytes or microglia. Rather, the primary effect of Tyro3 is upon the radial expansion of myelin. The loss of Tyro3 leads to a reduction in the number of myelin lamellae on axons, and is therefore most likely a key component of the regulatory mechanism by which oligodendrocytes match myelin production to axonal diameter.

INTRODUCTION

Neural signaling within the CNS depends upon efficient electrical impulse transmission along axons, an activity facilitated by compact myelin sheath which enables saltatory conduction [reviewed in (Snaidero and Simons, 2014)]. In the CNS, myelin is deposited by oligodendrocytes (OLs), and if myelin is damaged (demyelination) and the axon becomes exposed it causes saltatory conduction block and atypical signal transmission (Lee et al., 2012). If myelin is not properly re-established neurons become prone to damage and apoptosis (Meyer et al., 2001). Fortunately, healthy individuals have an innate ability to remyelinate following CNS insult; for reasons not entirely understood, remyelination eventually fails in the demyelinating diseases such as multiple sclerosis (MS) (Blakemore, 1974; Ludwin and Maitland, 1984; Barnett and Prineas, 2004; Etxeberria et al., 2016). MS is an autoimmune, demyelinating disease of the CNS, where re-established myelin is inappropriately thin (Périer and Grégoire, 1965; Prineas and Connell, 1979). Currently there are no therapeutic agents which can effectively induce or enhance remyelination in the CNS [reviewed in (Cole et al., 2017)]. While many approaches to enhance remyelination in MS have focused on promoting OPC migration to and differentiation at damaged sites, unfortunately less attention has been paid to understanding the regulation of myelin thickness both during development and repair (Franklin and Gallo, 2014). Regulation of the thickness, or radial diameter, of myelin is nevertheless a critical component of ensuring consistent conduction velocities within nerves. For example, in mice axonal diameter can range from <0.1 to >3μm within a single neuronal bundle, yet to maintain optimal conduction velocity these axons are proportionally ensheathed by myelin at a roughly equivalent *g*-ratio (the ratio of axonal diameter to myelin diameter) of ~0.7 (Hildebrand and Hahn, 1978). Furthermore, even subtle changes in *g*-ratios can significantly alter conduction velocities (Smith and Koles, 1970; Waxman, 1980; Baraban et al., 2016).

Recent studies have implicated the receptor Tyro3 as a potential component of the molecular machinery that regulates axonal ensheathment and the radial expansion of myelin. Tyro3 is a member of the TAM (Tyro3, Axl and Mertk) family of receptors, a distinct receptor tyrosine kinase sub-family with relatively restricted expression profiles in the developed CNS [reviewed in (Binder and Kilpatrick, 2009)]. Tyro3 is expressed by mature oligodendrocytes and we have previously shown that in the absence of Tyro3 developmental myelination is delayed (Akkermann et al., 2017). Interestingly, while the density of total myelinated axons normalizes with maturity in Tyro3 deficient mice, myelin thickness does not and these mice consistently display thinner myelin than their WT counterparts; a phenotype also observed in the peripheral nervous system (Miyamoto et al., 2015; Akkermann et al., 2017).

Given these findings, we hypothesized that the hypomyelinated phenotype observed in the absence of Tyro3 would not only be present widely within the unchallenged adult CNS but would also be apparent during remyelination. We therefore investigated the influence of the Tyro3 receptor on myelination, demyelination and myelin repair in the rostral and caudal regions of the CC using the cuprizone mouse model. We have verified our previous findings in the rostral region of unchallenged mice and extended these findings to the caudal CC, where Tyro3 also appears to regulate myelin thickness. We show that loss of Tyro3 is associated with an accelerated rate of loss of myelinated axons during cuprizone challenge and decreased myelin thickness during remyelination. We have found that while the loss of Tyro3 causes a hypomyelinated phenotype, it does so without influencing OPC maturation or OL/microglial densities. Rather, Tyro3 influences the radial expansion of myelin such that the loss of Tyro3 leads to a reduction in the number of myelin lamellae on axons, and is therefore most likely a key component of the regulatory mechanism by which OLs match myelin production to axonal diameter.

MATERIALS AND METHODS

Animals

Tyro3$^{-/-}$ C57Bl/6 mice were a gift from Prof. Greg Lemke (Salk Institute of Biological Studies, La Jolla, CA) (Lu et al., 1999). Mice were previously fully backcrossed onto the C57Bl/6 background and maintained in a specific-pathogen-free environment during all breeding and experimentation (Akkermann et al., 2017). All animal experiments were conducted according National Health and Medical Research Council Guidelines and approved by institution Animal Ethics Committee.

Induction of demyelination/remyelination

Demyelination was induced by feeding 8-10 week old mice powdered feed (Barastoc, Pakenham, Victoria, Australia) containing 0.2% (w/w) cuprizone (bis-cyclohexanone oxaldihydrazone) for 3 weeks as previously described (Binder et al., 2008). Remyelination cohorts were treated with 0.2% (w/w) cuprizone for 5 weeks and then returned to regular mouse chow to allow remyelination for either 0, 2, 4 or 10 weeks, as previously described (Binder et al., 2011). For each Tyro3$^{-/-}$ cohort, Tyro3$^{+/+}$ C57Bl/6 littermates [hereafter referred to as wild-type (WT)] underwent the same treatment and were used as controls. Each cohort was sex balanced.

Transmission electron microscopy

For transmission electron microscopic (TEM) evaluation of myelin, mice were anesthetized [100mg/kg sodium pentobarbitone (Virbac, New South Wales, Australia)] and perfused intracardially with phosphate buffered saline (PBS) followed by 4% TEM grade paraformaldehyde (PFA) in PBS. Brains were cropped sagittally from lateral to medial exposing the CC at the midline line. Tissue was then incubated in Kanovsky's fixative overnight at 4°C and stored in 0.1M sodium cacodylate prior to post-fixing in 1.5% potassium ferrocyanide and 1% osmium tetroxide (in ddH_2O) for 2h. Subsequently, samples were dehydrated 30 mins each in 50%, 70%, 90%, 95%, 100% ethanol and 100% acetone, incubated twice in 1:1 Spurrs resin/100% acetone (overnight and 2h), followed by incubation in 100% Spurrs resin and embedding in resin at 70°C overnight. Semi-thin sections (0.5µm) were cut to evaluate tissue quality and orientation. Representative samples were then chosen, ultrathin sections (90nm) were cut and contrasted using 0.15% uranyl acetate and lead citrate. Images were captured using a Jeol JEM-1011 Transmission Electron Microscope (Jeol Ltd., Akishima, Tokyo, Japan) at 6,000X magnification. Numbers of myelinated axons were counted (using NIH ImageJ, 2.0.0v) and expressed as numbers of myelinated axons/mm^2 ± SEM. Myelin thickness was measured by hand in ImageJ by substituting axonal fiber diameter from total myelin diameter. Myelinated axon counts and myelin thickness analysis was performed in 3-4 electron micrographs per animal, per region with 3-4 animals used per cohort.

To quantify myelin lamellae, high power TEM (50,000-200,000X magnification) was used on the same tissue as above. At least 50 myelinated axons per mouse (*n*=3/group) were captured, ImageJ was used to quantify myelin lamellae and fibre diameter for each axon.

Immunohistochemical analysis

Mice were anesthetized [100mg/kg sodium pentobarbitone (Virbac, New South Wales, Australia)] and perfused intracardially with PBS followed by 4% PFA in PBS. Whole brains were embedded in optimum cutting temperature compound (Sakura Finetek, Tokyo, Japan) and frozen in isopropanol, on dry ice. Brains were orientated coronally and 10µm sections cut at appropriate regions of the brain: rostral sections were as close to Bregma 0.98 as the series of sections would allow; and caudal from Bregma -2.18. Blocking [1 h at room temperature (RT)] and subsequent antibody incubations were performed in 10% normal goat or donkey serum/0.1% BSA/0.3% Triton X-100. Tissue was incubated with rabbit anti-Olig2 (AB9610, Merck Millipore, Billerica, MA, USA) and goat anti-PDGFRα (AF1062, R&D Systems, Minneapolis, MN, USA) or mouse anti-APC ([CC-1] ab16794, Abcam, Cambridge, MA) and rabbit anti-Iba1 (019-19741, Wako Chemicals USA, Richmond, VA, USA). Primary antibodies were incubated over night at RT followed by incubation with appropriate fluorophore-conjugated secondary antibodies for 1h at RT (Jackson ImmunoResearch, Baltimore, PA, USA). The final incubation included Hoechst 33342 (H1399, Invitrogen, Carlsbad, CA, USA) to visualize cell nuclei. Images were captured with a 40X objective, using a Carl Zeiss Axioplan inverted fluorescent microscope (Carl Zeiss, Thornwood, NY, USA). Cell densities were counted using Adobe Photoshop (Adobe Photoshop CC 2017, version 18.1.1, San Jose, CA, USA) and corpus callosum area measured with ImageJ. Densities are expressed as cells/mm^2 ± SEM.

Statistical analyses

All statistical analyses were performed using GraphPad PRISM 7 (GraphPad, version 7.0c, San Diego, CA, USA). Differences between genotypes at a single time point were assessed using student's *t*-tests. Differences between grouped data (genotypes and multiple time points) were assessed using two-way ANOVA followed by Holm-Sidak’s multiple comparisons *post-hoc* tests. Differences between myelin thickness/myelin lamellae quantity and axon diameter were assessed using linear regression. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Myelin thickness and myelin wrap number in the corpus callosum decrease in the absence of Tyro3

We have previously shown that the loss of Tyro3 leads to a reduction in the thickness of myelin in both the optic nerve and the rostral CC of unchallenged adult mice (Akkermann et al., 2017). We sought to validate and extend these findings to other white matter regions by determining whether the hypomyelinated phenotype seen in Tyro3$^{-/-}$ mice was also apparent in the caudal CC.

We used electron microscopy to quantify the number of myelinated axons in both the rostral and caudal regions of the CC of both WT and Tyro3 deficient mice (Fig.1A,B). Consistent with previous results (Akkermann et al., 2017), we observed no difference in the density of myelinated axons in the rostral segment of corpus callosi of WT and Tyro3 deficient mice (Fig.1C; 702079 ± 40435 vs. 757576 ± 66320 myelinated axons/mm^2 WT versus Tyro3$^{-/-}$ respectively; student's *t*-test, p=0.5). Furthermore, no significant difference was seen in the total number of myelinated axons within the caudal CC of WT and Tyro3 deficient mice (Fig.1D; 706483 ± 9989 vs. 765786 ± 184728 myelinated axons/mm^2 WT versus Tyro3$^{-/-}$ respectively; student's *t*-test, p=0.8).

In order to assess the influence of the Tyro3 receptor on myelin thickness we then performed morphometric analysis of the myelinated fibres, with the width of the myelin sheath plotted as a function of axon diameter. In both the rostral and caudal CC, the myelin sheath was on average ~10nm thinner in Tyro3 deficient mice compared to WT counterparts (Fig.1E, F; linear regression, p<0.0001). We then extended this analysis by assessing myelin ultrastructure under high power TEM. We found that the myelin membrane layers appeared appropriately compact in the Tyro3$^{-/-}$ mice relative to WT controls (Fig.1G,H). However, although myelin appeared normal in the Tyro3$^{-/-}$ mice, axons of equivalent diameter had, on average, one fewer myelin wrap compared to WT mice in the rostral CC (Fig.1I; linear regression, p=0.0005). Additionally, we found that the Tyro3$^{-/-}$ mice also had one fewer myelin wrap than WT counterparts within the caudal CC (Fig.1J; linear regression, *p*=0.0007). Taken together, these results confirm our previous data that Tyro3 is not essential for the initiation of myelination in the CNS. Conversely, these data strongly support a central role for Tyro3 in controlling the number of myelin lamellae formed by an oligodendrocyte.

The rate of demyelination is increased in the absence of Tyro3

We have previously shown that the rate of demyelination is accelerated in the rostral CC following the loss of the TAM receptor ligand, Gas6 (Binder et al., 2008). In order to determine whether this effect was mediated through Tyro3, we subjected mice to cuprizone challenge for 3 weeks to induce partial demyelination in both Tyro3$^{-/-}$ and WT mice (Hiremath et al., 1997). The number of myelinated axons was assessed in both the rostral and caudal regions of the corpus callosum using EM. Representative images of the rostral CC are shown in Fig.2A,B. In the rostral CC, we observed a 1.7-fold reduction in the total number of myelinated axons in Tyro3$^{-/-}$ mice relative to WT controls, indicating that the rate of demyelination was significantly increased in the absence of Tyro3 (Fig.2C; 300388 ± 45578 vs. 504757 ± 31749 myelinated axons/mm^2 in Tyro3$^{-/-}$ mice versus WT mice respectively; student's *t*-test, *p*=0.02). In contrast, in the caudal CC, no significant differences were observed in the number of myelinated axons numbers in Tyro3$^{-/-}$ compared with WT controls (Fig.2D; 232558 ± 11481 vs. 496723 ± 265697 myelinated axons/mm^2 in Tyro3$^{-/-}$ versus WT mice respectively; student's *t*-test, *p*=0.4).

Tyro3 deletion does not alter the response of microglia or oligodendrocyte lineage cells to demyelination

We next wished to determine if the differences observed in myelination and demyelination were the result of changes in either oligodendrocyte lineage cells or in the number of microglia, both in unchallenged mice as well as following cuprizone-induced demyelination. In order to examine this, a separate cohort of WT and Tyro3$^{-/-}$ animals was challenged with cuprizone for three weeks. We then determined the density of PDGFRα^{+ve}/Olig2^{+ve} OPCs, CC1^{+ve} OLs and Iba1^{+ve} microglial densities from both the rostral and caudal CC (Fig.3A).

Following 3 weeks of cuprizone challenge there was a substantial loss of CC1^{+ve} OLs (Fig.3D,E), and an increase in the number of PDGFRα^{+ve} OPCs (Fig.3B,C), as has previously been described (Matsushima and Morell, 2001). In addition, as expected, the number of IBA1^{+ve} microglia was substantially increased in response to cuprizone-induced demyelination in both the rostral and caudal corpus callosum (Fig.3F,G). However, we found no evidence for any differences in the numbers of each of these lineages in any region between WT and Tyro3$^{-/-}$ mice following cuprizone challenge or in unchallenged controls (two-way ANOVA, p>0.05). In addition, given that demyelination was altered in the rostral region of the CC, we tested whether the activation of Iba1^{+ve} microglia was altered in this region by co-staining for Mac-3. We observed no significant difference in the percentage of microglia which were activated during demyelination in the rostral CC (96.13% ± 1.027 vs. 97.32% ± 0.8933 activated microglia/mm^2 in WT mice versus Tyro3$^{-/-}$ mice respectively; student's *t*-test, *p*=0.4). Collectively, these observations strongly support a specific role of Tyro3 upon myelin integrity, unrelated to the survival or proliferation of oligodendrocyte lineage cells or the activation of microglia.

Recovery from cuprizone-induced demyelination is altered in the absence of Tyro3

We had previously shown that not only is there and increase in the kinetics of demyelination, but that remyelination is also delayed in this context (Binder et al., 2011). We therefore wished to investigate whether this latter effect was also contributed to by Tyro3. Given our data showing that loss of Tyro3 increases demyelination following 3 weeks of cuprizone, we chose a 5 week time-point of cuprizone challenge to provide equivalent nadir levels of demyelination in both WT and Tyro3$^{-/-}$ mice. Following 5 weeks of cuprizone challenge, mice were returned to a normal diet for either 2, 4 or 10 weeks, allowing comparison of the kinetics of remyelination between WT and Tyro3$^{-/-}$ mice (Fig.4A-H). In contrast to our observations in Gas6$^{-/-}$ mice, we found that the time course of remyelination is not altered in the absence of Tyro3, as the density of myelinated axons is equivalent across both the Tyro3$^{-/-}$ and WT mice at maximal demyelination and after 2, 4 and 10 weeks of cuprizone recovery in both the rostral and the caudal CC (Fig.4I,J; two-way ANOVA, $p>0.05$), indicating that the initiation of axonal ensheathment following myelin damage is not dependent upon Tyro3.

Subsequently, we undertook morphometric analysis of individual myelinated axons during recovery. Focussing on axons >2μm, in the rostral CC, we found myelin was on average ~20nm, ~50nm and ~30nm thinner in the absence of Tyro3 when compared with WT mice at 2, 4 and 10 week recovery time-points, respectively (Fig.5A, A-C; linear regression, $p<0.0001$ for each of the 2, 4 and 10 week time-points). Unexpectedly, in the caudal CC following 2 weeks of recovery, myelin appeared ~20nm thicker in the absence of Tyro3 compared to WT (Fig.5D; linear regression, $p=0.02$). However, this effect was transient such that after 4 weeks remyelination, myelin within the caudal corpus callosum was ~5nm thinner in the Tyro3$^{-/-}$ compared to WT mice (Fig.5E; linear regression, $p=0.0001$). In addition, following 10 weeks of recovery, the deficiency in the extent of myelin ensheathment in the Tyro3$^{-/-}$ mice within the caudal CC was comparable to that observed in the rostral CC, measuring ~25nm thinner than the WT mice (Fig.5F; linear regression, $p<0.0001$). In order to determine whether the decrease in myelin thickness was due to a reduction in the number of myelin wraps, we used high power TEM to quantify the number of wraps in the rostral CC following 10 weeks of recovery. We found that axons from Tyro3 deficient mice had fewer wraps than WT axons of equivalent diameter (Fig.5G; linear regression, $p=0.0402$), with the effect particularly pronounced for larger axons (>1.5μm) diameter. Therefore, consistent with our observations in developmental myelination, Tyro3 plays an important role in the re-establishment of appropriate myelin layering following myelin damage. Further, the effect of the loss of Tyro3 is heightened following myelin damage and subsequent recovery, with a greater difference in myelin thickness at all recovery time-points compared with unchallenged mice.

Tyro3 deletion does not alter the response of microglia or oligodendrocyte lineage cells to remyelination

We next sought to examine whether Tyro3 influenced the cellular response during remyelination. To investigate this, WT and Tyro3$^{-/-}$ animals were challenged with cuprizone for 5 weeks and assessed either at this time or after 4 weeks recovery. We then determined the density of PDGFRα^{+ve}/Olig2^{+ve} OPCs, CC1^{+ve} OLs and Iba1^{+ve} microglial cells from both the rostral and caudal CC. Representative images are shown in Fig.6A. As expected, the proportions of all cell types examined were robustly altered during recovery from demyelination (Fig.6B-G). However, consistent with our previous data from the demyelination phase, we found no evidence that the loss of Tyro3 influenced the proportion of OPCs, mature OLs or microglia in either the rostral or the caudal CC (two-way ANOVA, $p>0.05$). Given the rapid recovery of myelin following cuprizone withdrawal, OPC and mature OL densities were also assessed following 2 weeks of remyelination. No significant differences were detected in either OPC or mature OL densities between the Tyro3$^{-/-}$ and WT mice at the 2 week recovery time point in the rostral CC (Fig.6H, $p=0.12$), indicating that the decreased myelin thickness observed during remyelination is not a result of altered oligodendroglial survival.

DISCUSSION

This study has verified and extended upon, our previous findings establishing that Tyro3 influences developmental myelination by regulating myelin thickness, an effect identified in the optic nerve and within the rostral and caudal corpus callosum (Akkermann et al., 2017). The current work has identified that Tyro3 also regulates the tempo of demyelination and the nature of myelin repair during and after exposure to cuprizone challenge. Further, Tyro3 does not regulate axonal ensheathment during remyelination, but rather, is involved in the fine-tuning of the radial expansion of myelin.

One striking and consistent feature of this study was the relationship of Tyro3 to the number of myelin layers around any given axon, both during normal development and during recovery from a demyelinating event. In unchallenged adult mice, the absence of Tyro3 led to a reduction of ~10nm in myelin thickness equating with, on average, one fewer myelin wrap per axon in both the rostral and caudal CC. The reduction in the number of myelin lamellae was also observed following 10 weeks of recovery from cuprizone-induced demyelination, implying a cell-intrinsic defect in the ability of Tyro3 deficient oligodendrocytes to appropriate quantities of myelin. Conversely, there was no alteration in the periodicity of the major dense- or intraperiod-lines unlike, for example, the irregular myelin lamellae periodicity and compaction in proteolipid protein (PLP) hemizygous mutant mice (Klugmann et al., 1997). Taken together, these data suggest that the Tyro3 regulated, hypomyelinated phenotype was likely due to less overall myelin membrane deposition rather than a defect in myelin compaction.

Given the role we have demonstrated for Tyro3 in controlling the radial expansion of myelin, it is perhaps unsurprising that we found a similar role for Tyro3 during recovery from myelin damage induced by oral administration of cuprizone. Focusing on the rostral CC, Tyro3 deficient mice consistently show thinner myelin during remyelination compared with WT mice; a phenotype also reflected in the caudal CC after prolonged recovery, although it remains formally possible that at longer recovery time-points, myelin thickness in Tyro3 deficient mice would reach pre-challenge levels. This effect appears to be most prominent in larger caliber axons. Whereas unchallenged Tyro3$^{-/-}$ mice show an ~10nm reduction in myelin thickness, an even greater reduction in myelin thickness of ~30nm was observed in large caliber (>2.2µm) axons following 10 weeks of recovery, suggesting that signaling via Tyro3 may be particularly important in the remyelination response following myelin injury.

What might be the molecular mechanism underpinning this effect? Surprisingly little is currently known about the mechanism by which oligodendrocytes 'count' the number of wraps, and thus match myelin production to axonal diameter. The intracellular signaling molecules Erk1/2 and Akt have previously been shown to affect myelin thickness in the CNS (Flores et al., 2008; Ishii et al., 2012) and these pathways are potentially regulated via fibroblast growth factor receptor (FGFR) 2 (Furusho et al., 2012; 2017) and also via Tyro3 (Akkermann et al., 2017). However, it is not clear how this signalling is functionally linked to myelin production and the determination of how much myelin each axon receives. What is known is that the normal radial expansion of myelin is dependent upon the expression and local translation of MBP within the myelin sheath, and that in the absence of MBP the number of myelin lamellae is severely reduced (Rosenbluth, 1980; Simons and Trotter, 2007; Nawaz et al., 2013). Interestingly, we and others have previously shown that Gas6 can enhance expression of MBP from both human and rodent oligodendrocytes (Binder et al., 2011; O'Guin et al., 2013), and that this effect is lost when Tyro3 is absent (Akkermann et al., 2017). A parsimonious view of these observations is that Gas6/Tyro3 is fundamentally implicated in calibrating optimal myelin thickness through activation of Erk1 signaling and in turn, MBP expression, although more extensive work will be required to confirm this hypothesis.

Although the molecular machinery implicated in regulating the extent of myelin production remains to be fully elucidated, our data strongly supports a late-stage oligodendrocyte-specific mechanism. Consistent with our previous work examining developmental myelination, the hypomyelination phenotype observed in the absence of Tyro3 does not correlate with differences in OPC, OL or microglial densities, either during myelin injury or repair. This is consistent with other studies indicating that control of the radial expansion of myelin is independent of OPC proliferation and OL maturation (Flores et al., 2008; Ishii et al., 2012). In addition, Tyro3 has been found to be predominantly expressed by mature oligodendrocytes rather than OPCs, and in all probability exerts an influence upon myelin production via direct oligodendrocyte effect. However, given that Tyro3 is expressed on a subset of neurons in the CNS, formal exclusion of an axonal contribution to the effect will require conditional deletion of Tyro3 (Prieto et al., 2007).

Conversely, although Tyro3 appears to be a critical component of the mechanism by which oligodendrocytes regulate myelin production, it appears to be dispensable for initiation of myelination. We found no alteration the number of myelinated axons in the corpus callosum either in unchallenged mice, or during myelin repair. In contrast, in a prior study, we found that Gas6 deficient mice have fewer myelinated axons during recovery from cuprizone challenge, a result not reproduced in Tyro3 deficient mice (Binder et al., 2008). This disparity clearly suggests that the delayed remyelination observed in the absence of Gas6 is due to reduced signaling via either Axl or Mertk. In support of this hypothesis, Ray *et al.* (2017) showed that remyelination was delayed in a Gas6$^{-/-}$Axl$^{-/-}$ double knockout (DKO) mice following cuprizone-induced demyelination (Ray et al., 2017). In addition, the authors observed increased expression of a number of inflammatory cytokines, strongly implicating microglial dysregulation as the main cause of delayed remyelination in the DKO mice. In totality, these data suggest a clear delineation between the roles of Axl and Tyro3 in recovery from myelin damage, whereby Gas6 signaling via Axl is critical for regulation of the inflammatory response of microglia, whilst signaling via Tyro3 is essential for the production of the appropriate amount of myelin by the oligodendrocyte. Less clear at present is the contribution of the other TAM receptor, Mertk, as its role in response to myelin damage has yet to be explicitly tested, something which warrants further attention in the future particularly given that *MERTK* is a known susceptibility gene for MS (Ma et al., 2010; Binder et al., 2016).

Intriguingly, although Tyro3 deficiency did not alter the rate of remyelination following cuprizone challenge, demyelination was worsened in the absence of Tyro3. It is important to note that the cells most affected by cuprizone are mature OLs, which of the TAM receptors, only express Tyro3. The effects were of a similar order of magnitude, suggesting the majority of the effect is driven through Tyro3 expression on oligodendrocytes within the rostral CC. It remains unclear what is driving the regional differences in susceptibility to cuprizone-induced demyelination in the context of TAM receptor signaling deficiency. The phenotype of the Tyro3 KO mice during the demyelinative phase could reflect either accelerated demyelination or, alternatively, an effect consequent to the thinner myelination that the KO mice start with prior to the onset of demyelination. To distinguish between these possibilities will require either a detailed time-course or the development and interrogation of an inducible Tyro3 knockout.

From a translational perspective, an important aspect of this study is that the pro-myelinating effect of Tyro3 is widespread and enduring, not only in unchallenged mice but also after long-term (10 weeks) recovery. Interestingly the thinner myelin membranes identified predominantly amongst large caliber axons (>2μm) in the Tyro3 deficient mice compared to WTs, emulates the myelin deficiency observed in MS patients. This deficiency in myelin thickness will have significant consequences for conduction velocity, as predicted by both Hodgkin and Huxley equations and experimentally (Smith et al., 1979; Duncan et al., 2017). Moreover, it has been observed that when myelin structure is restored to a thickness equivalent to undamaged myelin, for example when OPCs are derived from neural precursor cells in the SVZ, normal conductivity is restored (Remaud et al., 2017). By implication, drugs that aim to promote the radial growth of myelin could have significant therapeutic potential in MS and related disorders of demyelination, enabling optimal saltatory conduction to be re-established. Our results suggest that Tyro3 is a cogent therapeutic target to achieve this goal. It is important to recognize however, that myelin thickness is only one parameter that influences conduction velocity. It will be therefore be important to confirm that conduction velocity can be improved through Tyro3 activation via direct empirical electrophysiological testing.

The results presented above highlight the importance of the Tyro3 TAM receptor in regulating developmental myelination, demyelination and myelin repair, specifically its role in fine-tuning myelin thickness in large caliber axons. In the absence of Tyro3, myelin appears thinner during development and during the course of remyelination. Tyro3 thus joins a limited list of receptors known to be involved in regulating the radial expansion of myelin in the CNS. Neurodegenerative diseases such as MS desperately need myelin reparative drugs to ameliorate the neural decline which causes physical disabilities. Due to the narrow expression profile of Tyro3 in the CNS and the pro-myelinating effect it exhibits, we propose Tyro3 as a potential target to improve myelin quality and hence functional outcome of repair after a demyelinating insult.

ACKNOWLEDGEMENTS

Grant sponsors: Genzyme (a Sanofi company) provided funding via an Investigator Sponsored Study. Genzyme was not involved in the design, collection, analysis or interpretation of study data. Genzyme was given the opportunity to review the manuscript and provide comment but the decision to submit the manuscript for publication was made by the authors independently. Multiple Sclerosis Research Australia via an incubator grant, Australian Government Research Training Programme Scholarship (to FB), Melbourne International Research Scholarship (to RA), Victorian Government (Florey Institute of Neuroscience and Mental Health), Operational Infrastructure Support Grant.

The authors thank Prof. Greg Lemke of the Salk Institute (San Diego) for the kind gift of the Tyro3 knockout mice. They also thank Mr Ashwyn Perera for technical assistance. The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1: Myelin thickness and myelin wrap number are reduced in the absence of Tyro3. (A,B) To assess the effect of Tyro3 on myelination, total myelinated axons/mm^2 and myelin thickness were quantified in both the rostral and caudal regions of the corpus callosum (CC) using EM. (C,D) No difference was observed in the number of myelinated axons in the rostral or caudal CC between the WT and Tyro3$^{-/-}$ mice. (E, F) Axon diameter and myelin thickness of nerve fibres were measured in both the rostral and caudal CC. Tyro3 deficient mice showed significantly thinner myelin in both the rostral and caudal segments of the CC compared with WT mice ($p<0.0001$, $p<0.0001$, rostral and caudal respectively). (G, H) Total myelin lamellae were quantified. Whilst Tyro3 deletion did not alter myelin compaction, an average of one fewer myelin lamella was observed in the absence of Tyro3 compared with WT axons of comparable diameter in both the rostral (I) and caudal (J) CC ($p=0.0005$, $p=0.0007$). Numerical results are presented as mean ± SEM, n=3-4 mice per group, statistical significance was assessed using Student's *t*-test for myelinated axon comparisons; linear regression was used for myelin thickness and lamellae comparisons. Scale bar = 2µm.

Figure 2: The rate of demyelination is increased in the absence of Tyro3. Demyelination was induced in wild-type (WT) and Tyro3$^{-/-}$ mice (*n*=3-4/group) by oral administration of cuprizone for three weeks. (A-D) To assess the effect of Tyro3 on demyelination, total myelinated axons/mm^2 and myelin thickness were quantified in both the rostral and caudal regions of the corpus callosum (CC) using EM. We observed a significant reduction in the number of myelinated axons within the rostral CC in the absence of Tyro3 compared with WT mice (E; $p=0.02$) but not the caudal region (F; $p=0.4$). Numerical results are presented as mean ± SEM, statistical significance was assessed using Student's *t*-test. Scale bar = 5µm.

Figure 3: Tyro3 deletion does not alter the response of microglia or oligodendrocyte lineage cells to demyelination. Wild-type (WT) and Tyro3$^{-/-}$ mice (*n*=3-4/group) were fed either a normal diet or feed containing 0.2% cuprizone for three weeks. Representative images of the corpus callosii are shown in panel (A). Immunohistochemistry was performed to assess the number of oligodendrocyte lineage cells (Olig2, magenta) and OPCs (PDGFRα, yellow) or mature oligodendrocytes (CC1, magenta) or microglia (IBA1, magenta). Nuclei were visualised by co-staining with Hoechst (cyan). Examples of positive cells are indicated by white arrow heads. (B, C) No difference in oligodendrocyte precursor cell densities was observed between Tyro3$^{-/-}$ and WT mice in either the rostral or caudal CC ($p=0.7$, $p=0.1$, rostral and caudal respectively). (D, E) No variation in oligodendrocyte densities was observed between Tyro3$^{-/-}$ and WT mice in either the rostral or caudal CC ($p=0.8$, $p=0.06$, rostral and caudal respectively). (F, G) Tyro3 deficiency did not alter microglial densities in either the rostral or caudal CC when compared to WT mice ($p=0.9$, $p=0.3$, rostral and caudal respectively). Numerical results are presented as mean cells/mm^2 ± SEM. Statistical significance was

assessed using two-way ANOVA with Holm-Sidak's multiple comparisons, post-hoc test. Scale bar =20µm.

Figure 4: Loss of Tyro3 does not alter the rate of axon re-ensheathment during recovery from cuprizone-induced demyelination. Demyelination was induced in wild-type (WT) and Tyro3$^{-/-}$ mice (*n*=3-4/group) by oral administration of cuprizone for five weeks. Mice were then returned to normal feed for either 0, 2, 4 or 10 weeks to allow remyelination to occur. To assess the effect of Tyro3 on remyelination, total myelinated axons/mm^2 and myelin thickness were quantified in both the rostral and caudal regions of the corpus callosum (CC) using EM at each time point. Representative images of WT (A-D) and Tyro3$^{-/-}$ (E-H) from the rostral region of the CC are shown. No significant difference was observed in total myelinated axons between WT and Tyro3$^{-/-}$ mice, in the rostral (I) or caudal (J) CC at 0 (p>0.9, p=0.8), 2 (p=0.4, p>0.9), 4 (p>0.9, p=0.3), or 10 (p>0.9, p>0.9) weeks recovery. Numerical results are presented as mean ± SEM. Statistical significance was assessing using two-way ANOVA with Holm-Sidak's multiple comparisons, post-hoc test. Scale bar =5µm.

Figure 5: Myelin sheath thickness is reduced during recovery from cuprizone-induced demyelination in the absence of Tyro3. Demyelination was induced in wild-type (WT) and Tyro3$^{-/-}$ mice (*n*=3-4/group) by oral administration of cuprizone for five weeks. Mice were then returned to normal feed for either 0, 2, 4 or 10 weeks to allow remyelination to occur. Axon diameter and myelin thickness of nerve fibres were measured after 2 (A,D), 4 (B, E) and 10 (C, F) weeks recovery in both the rostral (A-C) and caudal (D-F) corpus callosum. In the rostral region, myelin was thinner in the absence of Tyro3 compared with WT mice at 2, 4 and 10 weeks recovery (A-C; all *p*-values <0.0001). In the caudal region, when compared with WT mice, myelin was transiently thicker following 2 weeks of recovery (D; p=0.02). This effect was not enduring, as following 4 and 10 weeks of recovery myelin was significantly thinner in the caudal CC of Tyro3$^{-/-}$ mice than that of WT counterparts (E, F; p=0.001, p<0.0001, 4 and 10 weeks recovery respectively). (G) Fewer myelin lamellae were observed in the rostral CC following 10 weeks of recovery (p=0.0402). Statistical significance was assessed using linear regression.

Figure 6: *Tyro3 deletion does not alter the response of microglia or oligodendrocyte lineage cells to remyelination*. Demyelination was induced in wild-type (WT) and Tyro3$^{-/-}$ mice (*n*=3-4/group) by oral administration of cuprizone for five weeks. Mice were then returned to normal feed for either 0, 2 or 4 weeks to allow remyelination to occur. Representative images of the corpus callosii are shown in panel (A). Immunohistochemistry was performed to asses the number of oligodendrocyte lineage cells (Olig2, magenta) and OPCs (PDGFRα, yellow) or mature oligodendrocytes (CC1, magenta) or microglia (IBA1, magenta). Nuclei were visualised by co-staining with Hoechst (cyan). Examples of positive cells are

indicated by white arrow heads. (B, C) There were no significant difference in oligodendrocyte precursor cell densities between Tyro3$^{-/-}$ and WT mice in either the rostral or caudal CC (p=0.6, p=0.3, rostral and caudal respectively). (D, E) No difference in oligodendrocyte densities was seen between Tyro3$^{-/-}$ and WT mice in either the rostral or caudal CC (p=0.7, p=0.06, rostral and caudal respectively). (F, G) Tyro3 deficiency did not significantly alter microglial densities in either the rostral or caudal CC when compared with WT mice (p=0.8, p=0.2, rostral and caudal respectively). (H) No significant difference was observed in the density of either OPCs or mature oligodendrocytes in Tyro3$^{-/-}$ and WT mice following 2 weeks of remyelination in the rostral CC (*p=0.19, p=0.12* OPCs and mature oligodendrocytes respectively). Numerical results are presented as mean cells/mm^2 ± SEM. Statistical significance was assessed using two-way ANOVA with Holm-Sidak's multiple comparisons, post-hoc test. Scale bar =20µm.