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Drug discovery for remyelination and treatment of MS

Running title: Drug discovery for remyelination

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Main points:

Enhancing remyelination is a potential treatment for MS

Recent studies have identified numerous compounds that promote remyelination

New technologies will further accelerate MS drug discovery

Abstract

Glia constitute the majority of the cells in our nervous system, yet there are currently no drugs that target glia for the treatment of disease. Given ongoing discoveries of the many roles of glia in numerous diseases of the nervous system, this is likely to change in years to come. Here we focus on the possibility that targeting the oligodendrocyte lineage to promote regeneration of myelin (remyelination) represents a therapeutic strategy for the treatment of the demyelinating disease multiple sclerosis, MS. We discuss how hypothesis driven studies have identified multiple targets and pathways that can be manipulated to promote remyelination *in vivo*, and how this work has led to the first ever remyelination clinical trials. We also highlight how recent chemical discovery screens have identified a host of small molecule compounds that promote oligodendrocyte differentiation *in vitro*. Some of these compounds have also been shown to promote myelin regeneration *in vivo*, with one already being trialled in humans. Promoting oligodendrocyte differentiation and remyelination represents just one potential strategy for the treatment of MS. The pathology of MS is complex, and its complete amelioration may require targeting multiple biological processes in parallel. Therefore, we present an overview of new technologies and models for phenotypic analyses and screening that can be exploited to study complex cell-cell interactions in *in vitro* and *in vivo* systems. Such technological platforms will provide insight into fundamental mechanisms and increase capacities for drug-discovery of relevance to glia and currently intractable disorders of the CNS.

Introduction

Glia represent the majority of cells in our nervous system. Although long-cited to represent 90% of the cells in the human central nervous system (CNS), more recent estimates suggest that this may be closer to half (Bartheld, Bahney, & Herculano-Houzel, 2016). Whatever the numerical contribution of glia cells may be, advances in recent years have firmly established the fundamental roles of glial cells in regulating many aspects of nervous system formation, function, and dysfunction. The myriad of essential functions of oligodendrocyte progenitor cells (OPCs), oligodendrocytes, astrocytes and microglia in the CNS have been extensively reviewed elsewhere (Chung, Welsh, Barres, & Stevens, 2015; Freeman & Rowitch, 2013; Zuchero & Barres, 2015). Highlighting the increasing awareness of glial cells as viable therapeutic targets for the treatment of disease, *Glia* recently published a special issue called “Gliotherapeutics” (Möller & Boddeke, 2016a). In that issue Moeller and Boddeke provided an overview of the early stages of drug discovery projects, with a focus on molecular target-driven approaches most commonly employed in the pharmaceutical industry (Möller & Boddeke, 2016b). Additional articles focussed on specific molecular targets and their relationship to glia for disease intervention (Dale, Staal, Eder, & Möller, 2016; Garden & Campbell, 2016; Möller et al., 2016; Pearse & Hughes, 2016), and on emerging evidence that microglial play a major role in the pathogenesis of Alzheimer’s disease (Wes, Sayed, Bard, & Gan, 2016). Here we will focus on the concept that enhancement of the endogenous process of myelin regeneration (remyelination) represents a promising intervention for the treatment of multiple sclerosis (MS). Indeed, it is entirely possible that remyelination-enhancing medicines will come to represent the first class of drugs developed specifically to target glia, and the first to promote bona fide regeneration in the CNS. We will first focus on how hypothesis-driven studies have provided candidate pathways that can be manipulated to promote remyelination *in vivo*. We will then discuss recent hypothesis-generating phenotypic screens of small molecule compounds that identified novel regulators of oligodendrocyte biology and new entry-points for the enhancement of myelin regeneration. However, the pathology of MS is complex, and effective interventions may involve targeting multiple cell types and cell-cell interactions, and possibly doing so differentially over disease-course. Therefore, it is clear that integrated analyses of the role of distinct cell types and cell-cell interactions at the tissue, organ and whole animal level will be essential for gaining a fuller understanding of therapeutic options. As such, in the third part of this review, we take the opportunity to focus on emerging technologies that are not yet widely employed by glial cell biologists, but which are sure to augment both hypothesis-driven and translational/ drug discovery research in general.

Targeting the oligodendrocyte lineage for the treatment of disease

The fact that CNS myelin is disrupted in MS has been known for over 150 years, since the initial identification of the disease by Jean-Martin Charcot. However, in recent years it has become clear that oligodendrocytes (the myelin-producing cells of

the CNS) are affected in numerous neurodegenerative, neurodevelopmental and neuropsychiatric disorders. This includes disruption to oligodendrocytes and myelinated axons in Alzheimer's disease (Bartzokis, 2011), Huntington's disease (B. Huang et al., 2015), Motor Neuron disease (Kang et al., 2013), Downs syndrome (Olmos-Serrano et al., 2016), Autism (Pacey et al., 2013), neonatal hypoxia (Buser et al., 2012), and schizophrenia (Hercher, Chopra, & Beasley, 2014), in addition to numerous leukodystrophies characterised by widespread disruption to the white matter (Boespflug-Tanguy, Labauge, Fogli, & Vours-Barriere, 2008). Extensive fundamental research will be required to determine to what extent oligodendrocyte dysfunction plays primary or secondary roles in different conditions, and in which diseases targeting of the oligodendrocyte lineage may be rational. Therefore, for the purposes of this review, we will focus on the now rationale that enhancing the endogenous capacity of the CNS to regenerate myelin represents a viable option for disease intervention in MS.

The CNS exhibits an impressive endogenous capacity to regenerate myelin following its loss (demyelination). Remyelination has in fact, long been known to occur in MS (Prineas & Connell, 1979), and is also observed following numerous experimental modes of demyelination (e.g. (Blakemore, 1974; M. B. Bunge, Bunge, & RIS, 1961; Gledhill, Harrison, & McDonald, 1973; Reier & Webster, 1974)) and oligodendrocyte ablation (Gritsch et al., 2014; Locatelli et al., 2012; Pohl et al., 2011; Traka et al., 2010). The regeneration of CNS myelin is possible because of the existence of oligodendrocyte progenitor cells (OPCs, also called oligodendrocyte precursor cells or NG2 cells) that are distributed throughout the CNS (Dawson, Polito, Levine, & Reynolds, 2003). OPCs are capable of generating mature myelin-producing oligodendrocytes from early development well into adulthood in the healthy nervous system (E. G. Hughes, Kang, Fukaya, & Bergles, 2013a; Yeung et al., 2014; Young et al., 2013). In adults, new oligodendrocytes can be generated in response to learning (McKenzie et al., 2014; L. Xiao et al., 2016) and, importantly, also in response to demyelination (Tripathi, Rivers, Young, Jamen, & Richardson, 2010; Zawadzka et al., 2010). However, the regenerative process of remyelination is imperfect, declines in efficiency with age (Goldschmidt, Antel, König, Bruck, & Kuhlmann, 2009; Ruckh et al., 2012), and occurs to very varying degrees between lesions within individual MS patients and between different individuals (Hickey, 1999). Despite the complex pathologies of MS, the presence of OPCs with an impaired ability to differentiate into myelinating oligodendrocytes characterises a large proportion of MS lesions (A. Chang, Tourtellotte, Rudick, & Trapp, 2002; Kuhlmann et al., 2008; Lucchinetti et al., 1999; Ozawa et al., 1994; Wolswijk, 1998). Therefore, it is thought that interventions intended to overcome this bottleneck of oligodendrocyte differentiation may enhance myelin regeneration. But why might enhancing remyelination actually impact MS?

Multiple sclerosis is generally thought to be triggered by autoimmune-mediated attack of CNS myelin (Compston & Coles, 2008). Reflecting the inflammatory trigger of disease is the fact that essentially all currently-available frontline treatments for

MS target the adaptive immune system. Such treatments are now very effective in reducing the number and severity of debilitating immune-mediated attacks during the early relapsing-remitting stages of disease (Comi, Radaelli, & Soelberg Sørensen, 2016). However, these anti-inflammatory treatments have not yet been shown capable of preventing progression of disease beyond the relapsing-remitting stage to the later progressive stage of disease, which is characterised by extensive axonal degeneration (Trapp et al., 1998). There are currently no treatments available for the progressive stages of disease, which represents a huge unmet challenge. It is now thought that long-term demyelination contributes to the emergence of neurodegeneration seen in progressive stages of MS. Demyelination may lead to axonal and neuronal degeneration in multiple ways. First, demyelination leaves axons exposed to potentially damaging signals in the microenvironment, including those released by cells of the innate immune system (Mahad, Trapp, & Lassmann, 2015). Demyelination also leads to dysregulation of conduction (McDonald & Sears, 1969), and to the insertion of ion channels and transporters into the axonal membrane to maintain activity (Craner, Lo, Black, & Waxman, 2003; Craner et al., 2004; Prineas & Connell, 1979): such adaptations may inadvertently lead to large influxes of ions, such as the import of excessive and damaging amounts of Ca²⁺ into the axon (Franklin, Ffrench-Constant, Edgar, & Smith, 2012; Lassmann, van Horssen, & Mahad, 2012; Waxman, 2006). In addition, myelin provides trophic and metabolic support to axons (Fünfschilling et al., 2012; Nave & Trapp, 2008; Saab, Tzvetanova, & Nave, 2013), the disruption of which can in principle lead to axonal degeneration (Y. Lee et al., 2012b), and possibly also in MS. Such key roles of myelin in maintaining axonal health and function has led to the simple concept that promoting myelin regeneration in MS may not only help restore optimal circuit function, but also serve to protect axons and neurons from degeneration (Franklin et al., 2012). Indeed, experimental studies have validated the premise that remyelination helps restore conduction (K. J. Smith, Blakemore, & McDonald, 1979), and is neuroprotective (Irvine & Blakemore, 2008; Mei, Lehmann-Horn, et al., 2016a). Therefore, promotion of remyelination is considered as a promising therapeutic strategy for the treatment of MS (Franklin & Goldman, 2015). However, it is important to note that the pathology of MS is complex, and that promotion of remyelination is only one possible strategy for intervention, which we discuss below.

Hypothesis driven approaches and identification of strategies to promote myelin regeneration

In recent years, our understanding of the molecular mechanisms governing oligodendrocyte differentiation and myelination has increased dramatically, and is summarised in numerous reviews (Ackerman & Monk, 2015; Baraban, Mensch, & Lyons, 2016; Bercury & Macklin, 2015; Emery & Lu, 2015; Gaesser & Fyffe-Maricich, 2016; Galloway & Moore, 2016; Nave & Werner, 2014). Interestingly, although the contexts of developmental myelination and myelin regeneration are distinct, there is remarkable conservation in the cellular and molecular mechanisms by which oligodendrocytes differentiate and myelinate axons in both scenarios (Fancy, Chan,

Baranzini, & Rowitch, 2010; Franklin & Goldman, 2015). We will first focus on hypothesis driven studies that have identified regulators of remyelination with therapeutic potential (Table 1). See Box 1 for overview of the most commonly used laboratory models of demyelination referred to throughout this review. It is essential to note that no currently available animal model recapitulates the complex and divergent pathogenesis of MS. Individual experimental systems have complementary strengths and weaknesses in allowing dissection of specific biological processes associated with disease, e.g. adaptive immune response against myelin, or demyelination with subsequent remyelination (see Box 1). This means that translation of interventions of therapeutic potential from animal models to human represents a major challenge, as will be discussed briefly in the next section.

Lingo-1 and antibody based interventions

Myelin is inhibitory to axonal regeneration in the CNS, an effect mediated in part through the action of myelin-expressed NogoA on the regulation of RhoA activity in axons (Cao et al., 2010). NogoA signals through a receptor complex on the axon containing the Nogo-Receptor, and associated factors p75 and Lingo1 (Cao et al., 2010). Following its identification as a functional axonal receptor linking Nogo and RhoA-mediated growth arrest of axons during regeneration (Mi et al., 2004), Lingo1 was also found to be expressed by oligodendrocytes (Mi et al., 2005). This suggested the hypothesis that Lingo1 may also negatively regulate myelination through RhoA signalling in oligodendrocytes. Indeed, overexpression of Lingo1 impaired myelination, whereas abrogation of Lingo1 function accelerated oligodendrocyte differentiation *in vitro*. Furthermore, Lingo1 mutant mice exhibited premature oligodendrocyte differentiation and myelination (Mi et al., 2005). Key follow-up studies showed that antibodies directed against Lingo-1 could improve remyelination following both inflammatory (Mi et al., 2007) and Cuprizone-mediated (Mi et al., 2009) demyelination. In fact, humanised anti-Lingo 1 antibodies have been shown to exhibit a good safety profile in humans and have undergone phase II clinical trials for the treatment of MS. To date, the results of these trials appear inconclusive, perhaps reflecting the fact that the anti-Lingo1 trial represented the first ever trial that aimed to directly target remyelination. Patient stratification, treatment dosing, duration, and trial readouts remain important considerations for ongoing trial design for those that aim to assess how a potential remyelination-enhancing medicine might impact patients with MS (Koch, Cutter, Stys, Yong, & Metz, 2013; Plantone, De Angelis, Doshi, & Chataway, 2016). In particular, the ability to conclusively image remyelination in humans needs to be fully realised, either by MRI-based or PET ligand-based modalities, and is a topic of intense investigation (Absinta, Sati, & Reich, 2016; Bodini et al., 2016). Until we have the technologies to quantify remyelination in humans, and to assess associated functional output, our ability to determine the utility of remyelination-enhancing strategies will be difficult. Therefore, a major challenge in the field is to ensure that clinical trial design keeps pace with lab-based discoveries that generate leads of therapeutic potential.

Interestingly, antibodies against Lingo-1 are not the only ones shown to exhibit promising effects on myelin regeneration. Indeed, disruption to NogoR itself has also been shown to improve remyelination in a toxin-based model of demyelination (Chong et al., 2012), and anti-Nogo antibody based therapies have undergone phase I trials for prospective treatment of MS (Ineichen et al., 2017). The biology surrounding the Nogo-NogoR/Lingo1 is complex, (Schwab, 2010) as expression of Lingo1 in axons as well as oligodendrocytes can inhibit oligodendrocyte differentiation (X. Lee et al., 2007), highlighting possible bidirectional (axon-oligodendrocyte) signalling between Nogo (or other ligands) and the NogoR-Lingo1-p75 receptor complex. However, Lingo1 and Nogo remain viable drug target candidates for the treatment of disease. Indeed, in parallel Nogo/NogoR remains a target for treatment of spinal cord injury (Zörner & Schwab, 2010), where it is possible that the role of this signalling complex in regulating both axonal regrowth and remyelination may serve dual beneficial effects. Interestingly, another recombinant antibody (rHlgM22) originally derived from a patient with Waldenstrom's macroglobulinemia, has also been shown capable of promoting remyelination following both toxin (Bieber et al., 2002) and inflammatory-mediated (Pirko et al., 2004; Warrington et al., 2007) demyelination. Although the molecular mechanisms by which this antibody, rHlgM22, functions are unclear, its promise in pre-clinical studies has led to a phase I clinical trial for prospective treatment of MS (Kremer, Küry, & Dutta, 2015).

Retinoid X Receptor gamma

Focal toxin-induced demyelination of specific white matter tracts have proven very useful for understanding the biology of remyelination in animal models, given that the precise timing of demyelination and remyelination can be well defined (Blakemore & Franklin, 2008). For example, the distinct phases of the response to focal demyelination allowed Huang et al. to identify gene expression profiles that characterised distinct periods of remyelination. They hypothesised that genes differentially regulated between demyelinated and remyelinated lesions at specific stages might include candidate regulators of remyelination. Huang et al., isolated lesioned tissue at distinct stages following toxin-mediated demyelination of the rat cerebellar caudal peduncle and carried out microarray based analyses of gene expression. Focussing on factors most highly upregulated at the stage when remyelination was active, they identified the nuclear hormone receptor Retinoid X Receptor gamma as a candidate mediator of remyelination (J. K. Huang et al., 2011). They found that RXR γ was expressed in the oligodendrocyte lineage, and underwent a change in subcellular localisation upon oligodendrocyte differentiation. Interestingly, this nuclear to cytoplasmic localisation of RXR γ was severely disrupted in MS tissue. Additionally, chemical compounds known to inhibit RXR signalling were found to impair oligodendrocyte differentiation *in vitro*, while promoting its signalling led to increased oligodendrocyte differentiation. Most importantly, treatment with the pan-RXR agonist, (targeting α , β and γ RXR forms) 9-cis- Retinoic acid, accelerated remyelination in aged animals (J. K. Huang et al., 2011). This highlighted the

possibility of targeting RXR to promote remyelination for the prospective treatment of MS. Indeed, a clinical trial using another pan-RXR agonist, already approved for the treatment of cutaneous T-cell lymphoma (Pileri, Delfino, Grandi, & Pimpinelli, 2013), Bexarotene, is in preparation for an MS trial. Both 9-cis-Retinoic acid and Bexarotene are non-specific with respect to agonising RXR receptors α , β and γ , and may have side effects that limit their long-term use, which has driven studies to identify isoform-specific agonists of RXR subtypes (Heck et al., 2016). However, it is possible that pan-RXR activation may actually have beneficial effects in promoting remyelination. In addition to the possibility that its activation directly drives oligodendrocyte differentiation through agonising RXR γ , activation of RXR α in microglia may actively promote the clearance of myelin debris that results from demyelination (Natrajan et al., 2015). The efficient clearance of myelin debris is thought to represent another bottleneck to optimal regeneration of myelin (Kotter, Li, Zhao, & Franklin, 2006; Kotter, Setzu, Sim, Van Rooijen, & Franklin, 2001; Lampron et al., 2015; Safaiyan et al., 2016), as discussed later, and thus promoting RXR signalling may help to overcome two hurdles to efficient remyelination.

Wnt pathway regulation

Numerous studies have led to a working model that activation of the canonical Wnt signalling pathway serves to inhibit oligodendrocyte differentiation, and that over-activation of Wnt signalling may block efficient remyelination in disease (e.g. (Fancy et al., 2009; 2014; 2011; Rosenberg & Chan, 2009)). Indeed, small-molecule mediated inhibition of an enzyme (tankyrase) that stabilises a negative regulator of the pathway (axin2), promotes remyelination following toxin-mediated demyelination (Fancy et al., 2011). However, the roles of Wnt signalling in CNS (re)myelination are complex and potentially divergent between distinct regions, over time, and in the context of interactions with other cells and tissues (Guo et al., 2015; Yuen et al., 2014). Furthermore, the Wnt pathway is central to regulation of numerous biological processes (e.g. (Bielen & Houart, 2014; Lambert, Cisternas, & Inestrosa, 2016)) and the consequences of its systemic targeting over prolonged periods remain unclear. Therefore, despite the fact that numerous small molecule regulators of the Wnt pathway are available (B. Lu, Green, Farr, Lopes, & Van Raay, 2016) and represent viable options for life-threatening conditions (Tai et al., 2015), their utility for treatment of a life-long condition such as MS warrants careful consideration.

Manipulation of a number of other broadly expressed factors have also been shown capable of promoting remyelination *in vivo*, including interaction of the cholesterol-like molecule olesoxime with the Mitochondrial permeability transition pore (Magalon et al., 2012), and the small molecule inhibitor rolipram with Phosphodiesterase-4 (Syed et al., 2013) (Table 1). The precise mechanisms of action of these compounds on oligodendrocyte biology remain unclear, and thus whether the effects on remyelination are direct or indirect is unknown.

G-protein coupled receptors as targets to promote remyelination

A highly desirable attribute of a druggable target is cell type-specific expression in the target cell or tissue of interest. Cell type-specific expression of a druggable target reduces concerns over unwanted effects of compounds, e.g. those targeting core pathways shared across multiple cells and tissues. Therefore, goals to identify genes with cell type-specific expression in oligodendrocytes should in principle lead to more tractable targets for long-term manipulation of remyelination, where treatment may be required over decades in a disease like MS.

The transcription factors *olig1* and *olig2* are core regulators of the specification of oligodendrocyte lineage cells, with *olig1* playing a specific and critical role in regulating the timing of myelination and remyelination (Meijer et al., 2012). Based on the premise that factors under the control of *olig1* might identify targets that regulate myelination and remyelination, Chen et al., carried out microarray-based profiling of gene expression in *olig1* mutant mice. They found that the G-protein coupled receptor GPR17 was downregulated in expression by over 200-fold (Chen et al., 2009). Given that GPCRs represent excellent drug targets (Jacobson, 2015; Miao & McCammon, 2016), the role of this candidate factor in regulating myelination was investigated. Expression analysis indicated that GPR17 was largely restricted to the oligodendrocyte lineage, and it was found that overexpression of GPR17 inhibited oligodendrocyte differentiation, whereas its abrogation accelerated oligodendrocyte differentiation and thus myelination (Chen et al., 2009). Importantly, a recent study indicated that pharmacological inhibition of GPR17 using pranlukast accelerated oligodendrocyte differentiation following toxin-mediated demyelination, as did genetic abrogation of GPR17 (Ou et al., 2016). However, pranlukast does not specifically act on GPR17 (Grossman et al., 1997), but identification of a specific antagonist of GPR17 may represent a viable therapeutic option for enhancement of remyelination in the future.

Several GPCRs are now known to regulate myelinating glial cell development (Mogha, D'Rozario, & Monk, 2016). For example, GPR37 was recently identified as a potent negative regulator of oligodendrocyte differentiation and myelination. Microarray-based profiling of isolated cells identified GPR37 as strongly enriched in the oligodendrocyte lineage. Highlighting the cell type-specific function of GPR37 *in vivo*, constitutive knockout of this GPCR lead to a very specific phenotype in CNS myelination. GPR37 mutant mice exhibit premature oligodendrocyte differentiation, precocious myelination, and hypermyelination (H.-J. Yang, Vainshtein, Maik-Rachline, & Peles, 2016). Although its role in remyelination has not yet been reported, it is possible that modulation of GPR37 function could affect multiple stages of myelin regeneration. In addition to regulating oligodendrocyte differentiation, abrogation of GPR37 function could also impact the extent of myelination itself, which as we will discuss in detail in a later section is an aspect of remyelination that has received comparatively little attention to date. Interestingly, GPR37 is implicated in other diseases of the CNS, including Parkinson's disease

(Murakami et al., 2004) and Autism (Fujita-Jimbo et al., 2012; Tanabe, Fujita-Jimbo, Momoi, & Momoi, 2015), possibly due to regulation of dopaminergic signalling in neurons (Marazziti et al., 2007). Given its strong enrichment in expression in the oligodendrocyte lineage, it will be interesting to determine to what extent, if any, its functional roles in oligodendrocytes are relevant to these conditions.

GPR37 is closely related in structure to another GPCR, the endothelin receptor B (EdnrB) (N. J. Smith, 2015). Interestingly, pharmacological activation of EdnrB signalling can promote remyelination in an explant slice culture system, most likely by regulating the extent of myelination by oligodendrocytes (Yuen et al., 2013). However, antagonism of both EdnrA and EdnrB by a pan-antagonist can promote remyelination *in vivo* (Hammond et al., 2014), by promoting oligodendrocyte differentiation. It is possible that different endothelin receptors play distinct roles in oligodendrocyte differentiation (A) and myelination (B), and that targeting with receptor specific antagonists (A) and agonists (B) could synergistically improve remyelination. The role of another GPCR, kappa-Opioid receptor, in regulating remyelination will be discussed below in the context of compounds identified following an unbiased phenotypic chemical screen that aimed to identify regulators of GPCRs that affected myelination.

Gli1

The signalling molecule sonic hedgehog (Shh) is a major regulator of many biological processes (Ferent & Traiffort, 2015), and activation of the hedgehog (HH) pathway in target cells activates Gli transcription factors. Despite being a gold-standard readout of HH pathway activity, and associated with glioma (Rossi et al., 2011), the functional role of Gli1 in normal development and function of the nervous system remained elusive (e.g. (H. L. Park et al., 2000)) until recently. Given that previous studies had implicated neural stem cells (NSCs) in the sub-ventricular zone as potential contributors to remyelination (Nait Oumesmar, Picard-Riera, Kerninon, & Baron-Van Evercooren, 2008), Samanta et al., investigated the behaviour of HH-responsive NSCs to demyelination. To do so they generated a reporter mouse based on Gli activity to fate map the response of this subpopulation of NSCs to cuprizone-induced demyelination of the corpus callosum. They found that the HH-responsive subpopulation of NSCs responded significantly to demyelination by generating OPCs that migrated to sites of demyelination and subsequently differentiated into oligodendrocytes that contributed to remyelination (Samanta et al., 2015). Somewhat surprisingly, both genetic and small molecule-mediated inhibition of Gli1 itself promoted migration of OPCs to demyelinated axons, differentiation at lesions, and thus remyelination. Interestingly, this role appeared specific to inhibition of Gli1 and of a role in HH signalling, because inhibition of canonical HH signalling did not affect remyelination by NSCs (Samanta et al., 2015). Thus, despite the generality of HH signalling, this surprisingly specific function of Gli1 may form the basis of small molecule-based targeted therapies to enhance remyelination. This is further substantiated by observations that Gli1 expression may be transiently upregulated in

MS lesions by inflammation, potentially impairing regeneration of myelin (Y. Wang, Imitola, Rasmussen, O'Connor, & Khoury, 2008).

Phenotypic screens to discover mechanisms to promote remyelination

In parallel to the success of hypothesis driven studies in generating new targets and compounds to promote myelin regeneration, a set of recent studies have taken an unbiased phenotype-driven approach to identifying chemical modulators of remyelination. Phenotypic screens are enjoying something of a renaissance in terms of projects aimed at identifying interventions to treat diseases of the CNS (Prior et al., 2014; Pruss, 2010). Indeed, eight studies to date have taken the broadly similar approach of screening compound libraries for their ability to promote the differentiation of oligodendrocytes *in vitro* (Deshmukh et al., 2013; Joubert et al., 2010; Lariosa-Willingham et al., 2016; Mei et al., 2014; Mei, Mayoral, et al., 2016b; Najm et al., 2015; Peppard et al., 2015; Porcu et al., 2015) (Table 2). The results of these studies have already generated new compounds and targets of great promise (see Tables 1 and 2). Here we will discuss some of the salient issues regarding screen design and execution, and then examine in detail the hits identified in these screens for their ability to promote remyelination *in vivo*.

The key technical differences between the eight *in vitro*-based screens published to date can be categorised into four main areas, chemical library choice, cell source and type, assay readout, and secondary hit confirmation.

Chemical library choice

The choice of chemical library is an essential part in the design of a phenotypic screen. This challenge is quite distinct from that of library design and selection in more traditional drug discovery projects that focus on a specific molecular target (Balakin, Kozintsev, Kiselyov, & Savchuk, 2006). For phenotypic screens there are advantages to using small and well characterised libraries of compounds, including those with drugs already approved for use in man. The compounds in such libraries often have extensive information on their molecular target(s), meaning that one can move towards mechanistic studies of target function more easily. Furthermore, if a hit compound is already approved for treatment of another condition, a wealth of information about its safety profile and function is available (Pruss, 2010). This may allow for the rapid translation of findings into clinical trials with the aim of repurposing compounds for the treatment of a new disease, as has been shown for one of the hit compounds from the following set of screens (clemastine, see below). However, such an approach is ultimately limited in scope, as the relatively small number of such compounds means that the same compound(s) and compound classes will be identified when interrogating any biological process of interest. Therefore, an additional approach is to screen larger and more chemically diverse libraries to increase the chance of identifying new hits. This approach requires screening assays that are scalable, and robust methods for target deconvolution to identify the

mechanistic action of hit compounds. Interestingly, despite starting with different libraries, there has been some degree in overlap of compounds and target classes identified in the eight phenotypic screens for oligodendrocyte differentiation carried out so far, increasing confidence in reproducibility.

Cell-type

Of the eight *in vitro* cell-based studies screening for compounds that increase OPC differentiation, four used primary rat OPCs, one used mouse epiblast stem cell-derived OPCs and three used immortalised OPC cell lines from either rat or mouse. Perhaps the most important difference is the use of the primary cell lines in the Deshmukh, Mei, and Lariosa-Willingham studies, compared with immortalised lines used by Joubert, Peppard and Porcu. It has been known for over thirty years that primary OPCs can differentiate into oligodendrocytes in culture in the absence of neurons, (e.g. (Knapp, Bartlett, & Skoff, 1987; Zeller, Behar, Dubois-Dalcq, & Lazzarini, 1985)), even to the point of expressing differentiated markers of myelin and extending myelin-like membrane sheets (Simons, Krämer, Thiele, Stoffel, & Trotter, 2000). This default ability of oligodendrocytes to differentiate has enabled screens using primary oligodendrocytes to be carried out with the knowledge that such cells recapitulate many aspects of physiological development. Immortalised cell lines have the advantage of being relatively homogenous and rapidly expandable to quantities required for high-throughput screening, but how closely they reflect the biology of the oligodendrocyte lineage *in vivo* is more questionable. For example, the *Oli-neu* line (used by Joubert et al.) does not mature sufficiently to robustly express markers of myelination (see review by (Pereira, Dobretsova, Hamdan, & Wight, 2011)). Therefore, Joubert et al. quantified total actin-positive process length as a proxy of differentiation (Joubert et al., 2010). Porcu et al. also used the *Oli-neu* cell line, but in an attempt to overcome its limitations, overexpressed the transcription factor MyRF to drive myelin gene expression (Bujalka et al., 2013; Emery et al., 2009), prior to their screen (Porcu et al., 2015). Representing an important additional approach for the future, human oligodendrocytes with physiologically relevant properties can now be derived from both embryonic stem (ES) (Stacpoole et al., 2013) and induced pluripotent stem (iPS) cells (Livesey et al., 2016) and generated in sufficient numbers for screens. In addition, compounds showing efficacy in a human system would represent particularly promising candidates for prioritisation.

Assay design and readout

In any chemical screen, the ability to identify true hits whilst minimising false positive or false negative hits is of utmost importance. All screens will identify some false positives and negatives, however, the acid test of a screen's success is ultimately determined by how many hits turn into viable leads with translational potential. For a screen to be successful, it must begin with an appropriate assay design, including a robust and minimally biased readout method. Each of the eight *in vitro* OPC differentiation screens employed some form of automated analysis. Six of the eight screens were carried out using simple oligodendrocyte-enriched 2-D cultures grown

in multi-well plate format, with automated image acquisition (Deshmukh et al., 2013; Joubert et al., 2010; Lariosa-Willingham et al., 2016; Najm et al., 2015; Peppard et al., 2015; Porcu et al., 2015). Subsequent image analyses included relatively simple quantification of DAPI-positive nuclei surrounded by MBP staining, as used by Deshmukh et al., to serve as a proxy for oligodendrocyte differentiation. In contrast, both Najm et al., and Lariosa-Willingham employed more complex analyses of cell morphology to estimate myelin produced per MBP-expressing oligodendrocyte, in addition to assessing differentiation. Finally, for the Joubert and Peppard screens, cellular morphology alone was quantified, which in the case of the Peppard screen led to the identification of 1636 primary hits from a library of 73,000 compounds. This assay was performed without replicates, which together with the assay focussing on morphology alone as a readout, led to a high rate of false positives, as reflected by the fact that only 22 compounds ultimately demonstrated selective phenotypes on primary OPCs (Peppard et al., 2015). Therefore, initial screen design can help to identify meaningful hits and prevent laborious follow-up triaging studies.

The two screens carried out by Mei et al employed a unique assay to identify changes in OPC differentiation, by exploiting the ability of oligodendrocytes to myelinate inert objects (Bechler, Byrne, & Ffrench-Constant, 2015; S. Lee, Chong, Tuck, Corey, & Chan, 2013; S. Lee et al., 2012a). In an elegant application of this observation, Mei et al. created a 3D array of conical fused silica micropillars arranged within a 96-well format, such that the micropillars acted as substrates for rat OPCs to differentiate upon and also for oligodendrocytes to ensheath with myelinating processes (Mei et al., 2014). By acquiring two-colour confocal sections through the pillar array, the team were able to quantify the relative proportion of pillars surrounded by distinct rings of MBP-positive myelin membrane compared to PDGFR α -positive OPC processes. The relative number of pillars positive for either MBP or PDGFR α was used to assess the effect of compounds relative to untreated controls (e.g. more MBP rings suggests more differentiation, more PDGFR α rings suggests higher proportion of OPCs). Thus, this two-colour fluorescence-based assay called the Binary Indicant for Myelination using Micropillar Arrays, or BIMA (Mei et al. 2014), allowed automated quantitation ideally suited to large-scale screening that could readily identify and rank hits with a deceptively simple readout.

Hit confirmation and triaging:

Following hit identification from a chemical screen, the next step is to confirm and prioritise hits for follow-up. Validating and characterising hit function within the primary assay through increasing replicate number, performing simple concentration series and assessing possible non-specific cytotoxicity are simple methods to triage hits (Pruss, 2010). If hits have come from well-annotated libraries, then their putative molecular targets can be assessed using either genetic manipulations, or more simply by analysing the effects of additional compounds that hit the same target. Ultimately, to test whether compounds identified in a cell-based screen might have therapeutic potential, validation in more complex (ideally multiple) experimental

platforms is required. Such validation approaches were reported in the studies of Deshmukh, Najm and both of those by Mei et al. to the point demonstrating efficacy on remyelination in multiple *in vivo* models.

Hits from phenotypic chemical screens validated *in vivo* to enhance remyelination

Validating the effects of compounds identified from isolated cell culture systems *in vivo* is essential to prioritise hits for translation. Of the 159 hits identified in the eight *in vitro* OPC differentiation screens, 5 have been shown to improve remyelination *in vivo* thus far.

Muscarinic acetylcholine receptor antagonists, benztropine and clemastine

Given the degree of overlap in libraries screened in the studies carried out to date, it is no great surprise that they have many hit compounds and target classes in common. Among the most consistent target class identified across all the chemical screens were muscarinic acetylcholine receptors (mAChRs), with antagonists for mAChRs found as hits in 4 of 7 screens (Deshmukh et al., 2013; Mei et al., 2014; Mei, Mayoral, et al., 2016b; Najm et al., 2015). In their screen, Deshmukh et al. identified six anti-cholinergics; benztropine, clemastine, disopyramide, trospium, diacetyl-monoxime and homatropine, as positive regulators of OPC differentiation. They chose to follow up benztropine due to its ability to cross the blood brain barrier (BBB), oral bioavailability, and FDA approval. Benztropine is a drug used to manage the symptoms of Parkinson's disease (Friedman et al., 1997), and so a compound whose safety profile was already well known, thus representing a promising repurposing candidate for the treatment of MS. As with many compounds, benztropine acts on multiple targets and is known to block dopamine re-uptake and antagonise the H1 histamine receptor (Agoston et al., 1997; McKearney, 1982). However, Deshmukh et al., indicated that the pro-differentiation effect on OPCs were likely due to antagonism of mAChRs, through a series of co-treatments with modulators of mAChR, dopamine and H1 function. Importantly, benztropine also improved remyelination *in vivo*, and increased oligodendrocyte differentiation following cuprizone-mediated demyelination, where treatment led to significantly increased oligodendrocyte numbers and more myelin as assessed by luxol fast blue staining (Deshmukh et al., 2013). In addition, benztropine improved clinical outcome in a relapsing-remitting EAE mouse model, where it also increased numbers of mature oligodendrocytes and myelination, the latter assessed more definitively by electron microscopy (Deshmukh et al., 2013).

In agreement, Mei et al. (2014) found that a group of mAChR antagonists increased the percentage of MBP-positive rings in their BIMA assay (Mei et al., 2014). This group of hits correlated remarkably with those identified in the Deshmukh et al. screen, with clemastine and benztropine appearing in both. Mei et al. followed up clemastine, an FDA approved over the counter anti-histamine with an excellent

toxicity profile and CNS penetrance (Nicholson, 1985). Similar to benztropine, clemastine improved remyelination *in vivo*, using a lysolecithin-induced focal spinal cord demyelination mouse model (Mei et al., 2014). A follow-up study also showed that clemastine improved clinical score and remyelination in EAE, and, importantly, that such remyelination was neuroprotective (Mei, Lehmann-Horn, et al., 2016a). Furthermore, oligodendrocyte specific knockout of the M1 muscarinic receptor phenocopied the effects of clemastine treatment in the EAE model, indicating that clemastine mediates its effects on remyelination through this receptor (Mei, Lehmann-Horn, et al., 2016a). In a separate study, it was shown that primary human OPCs (hOPCs) also express the M3 muscarinic receptor, and that treatment with the M3-selective antagonist soligenacin could increase differentiation of isolated hOPCs (Abiraman et al., 2015). Furthermore, solifenacin appeared to increase the differentiation of hOPCs transplanted into shiverer/ mbp mutant mice, providing further evidence that antagonism of muscarinic receptor function may promote remyelination (Abiraman et al., 2015). In fact, given the excellent safety profile of clemastine, its potential for repurposing for MS is already under investigation in two clinical trials (optic neuritis and MS, clinical trial references: NCT02040298 and NCT02521311, respectively). Intriguingly, in another animal model study, clemastine was found to rescue behavioural deficits and aberrant prefrontal cortex myelination found in socially isolated mice (Liu et al., 2016), suggesting that promotion of myelination could in principle be used for the treatment of additional disorders.

κ -opioid receptor agonist (\pm)-U-50488

Given the previously mentioned suitability of GPCRs as drug targets, Mei et al. carried out a second screen focusing on GPCR agonists and antagonists using their elegant BIMA assay. From a library of 280 compounds, they identified a cluster of 12 κ -opioid receptor (KOR) agonists (out of 15 hits), which promoted OPC differentiation (Mei, Mayoral, et al., 2016b). KORs are expressed in post-mitotic oligodendrocytes and the best KOR agonist identified in their assay, (\pm)-U-50488, increased the proportion of MBP-positive cells in a purified OPC culture system, and promoted myelination in OPC-DRG myelinating co-cultures (Mei, Mayoral, et al., 2016b). They also showed that the (\pm)-U-50488 driven increase in OPC differentiation in culture was diminished using a KOR antagonist, and in mice lacking the KOR. Importantly, Mei and colleagues also demonstrated that (\pm)-U-50488 improved remyelination following toxin-induced demyelination and that this effect on remyelination was absent in an oligodendrocyte-specific KOR knockout mouse, providing further evidence of a direct effect of the compound on the oligodendrocyte lineage (Mei, Mayoral, et al., 2016b). Further emphasising the potential of targeting KOR for the treatment of MS was the parallel observation that (\pm)-U-50488 could ameliorate symptoms of EAE (Du et al., 2016).

Glucocorticoid receptor agonist clobetasol and miconazole acting on an unknown target

Two compounds from the Najm screen were followed up in detail, clobetasol and miconazole, both shown to be effective at increasing oligodendrocyte differentiation in a lysolecithin-induced mouse spinal cord demyelination model (Najm et al., 2015). Both clobetasol and miconazole were also shown to significantly improve the clinical outcome of mice with a form of EAE that aims to model chronic progressive MS (Najm et al., 2015). To identify the downstream targets of these compounds and deduce their mechanisms of action, Najm et. al. checked whether the compounds acted on mAChRs, before checking an array of 414 kinases, but found no changes in activity with clobetasol and miconazole treatment. Next, they performed RNA sequencing and phospho-proteomic analysis of treated versus untreated mouse OPC cultures. In doing so, they found that clobetasol upregulated genes downstream of the glucocorticoid receptor. Following on from this they showed that the glucocorticoid receptor was phosphorylated upon treatment with clobetasol, and that clobetasol-induced OPC differentiation, could be blocked by the glucocorticoid receptor antagonist RU486 (Najm et al., 2015). Shortly after the Najm screen was published, Porcu et al. also identified clobetasol from their screen in *Oli-neuM* cells (Porcu et al., 2015). Clobetasol, as well as a group of related glucocorticoids had previously been shown to act as agonists for the Smoothed receptor (J. Wang et al., 2010), and so Porcu et al. investigated whether its action in regulating HH signalling was involved in the pro-differentiation effect of clobetasol. By co-treating OPC cultures with clobetasol and smoothed antagonists itraconazole or cyclopamine, they showed that the increase in clobetasol-driven OPC differentiation *in vitro* was significantly reduced (Porcu et al., 2015), suggesting that this may be a complementary mechanism to the agonism of glucocorticoid receptors. It is important to note that clobetasol was also shown to reduce the severity of a relapsing-remitting EAE model when administered prophylactically, with this effect attributed to its action on T-cells (Najm et al., 2015). Therefore, clobetasol may play roles in multiple cell types and through different targets in biological processes of relevance to MS.

The molecular profiling analysis by Najm et al. indicated that miconazole regulates myelination by increasing phosphorylation of proteins in the mitogen-activated protein kinase (MAPK) pathway, specifically through phosphorylation of Erk1 and Erk2 (Najm et al., 2015). Interestingly, Erk1 and Erk2 have recently been shown to profoundly regulate the amount of myelin generated by oligodendrocytes (reviewed by (Gonsalvez, Ferner, Peckham, Murray, & Xiao, 2015), suggesting the possibility that miconazole may also increase myelination per se. However, given the broad roles of the MAPK pathway in multiple cells and tissues (Atay & Skotheim, 2017), it is unlikely that its systemic regulation represents a viable long-term treatment option in MS.

In summary, recent cellular-based phenotypic screens have identified numerous compounds and targets that can promote remyelination *in vivo*, one of which has

already led to a drug repurposing trial for the treatment of MS. Indeed, additional compounds, with roles in remyelination identified in other studies, are also currently being considered for repurposing for MS, including the anti-psychotic quetiapine (Y. Zhang et al., 2012). Interestingly, among its many prospective targets are muscarinic acetylcholine receptors, but the side-effects and numerous targets of quetiapine (Garver, 2000) limits its attraction for treatment of MS. Indeed, the fact that drugs are already approved for other conditions means, by definition, that they have effects outwith those on the oligodendrocyte lineage. Therefore, it is hard to assess at present how many other drugs already approved for use in humans have true potential for repurposing to promote remyelination and long-term treatment of MS. However, with only a small number of screens carried out so far, significant success has been made, and new target classes have been identified. Further assessment of current hits and identification of new hits from additional screens are sure to provide additional options for the targeted improvement of oligodendrocyte differentiation therapeutically. However, as we will discuss next, promoting oligodendrocyte differentiation is just one strategy to enhance remyelination.

Additional strategies to improve remyelination

Promoting recruitment of oligodendrocyte progenitor cells to sites of demyelination

We have focussed thus far on how promoting oligodendrocyte differentiation may represent a potential treatment for MS. This is based on the observation that many MS lesions contain OPCs in sufficient abundance, but that there is an apparent failure of these cells to differentiate into oligodendrocytes (A. Chang et al., 2002; Kuhlmann et al., 2008; Lucchinetti et al., 1999; Ozawa et al., 1994; Wolswijk, 1998). However, many MS lesions completely lack OPCs, indicating that their recruitment to lesions represents another bottleneck to regeneration (Boyd, Zhang, & Williams, 2013). OPCs are present in the CNS throughout life (Dawson et al., 2003) and their number and distribution appears to be homeostatically controlled. For example, in both the zebrafish spinal cord and mammalian cortex, individual OPCs exhibit dynamic behaviour whereby they extend and retract processes that interact with those of adjacent OPCs. When the processes of neighbouring OPCs interact they withdraw from one another indicating that repulsive interactions regulate their distribution (E. G. Hughes, Kang, Fukaya, & Bergles, 2013b; Kirby et al., 2006). Interestingly, when a small number of OPCs are ablated, neighbouring OPCs divide and migrate to fill the void (E. G. Hughes, Kang, Fukaya, & Bergles, 2013b; Kirby et al., 2006). Homeostasis of OPCs can also be seen at the wider population level. Despite impairing the proliferation of 90% of adult OPCs through genetic targeting, it was recently observed that the remaining non-recombined cells could compensate and proliferate such that total OPC number was maintained at the normal level (Schneider et al., 2016). This homeostatic propensity of OPCs indicates that this cell population is tractable for expansion and mobilisation to MS lesions where they are

absent. However, there are numerous environmental features that may limit OPC recruitment to MS lesions. It has been shown that expression of semaphorins known to regulate developmental OPC migration (Sema 3A and 3F) are differentially upregulated in distinct MS lesions (Williams et al., 2007). Experimental studies indicate that Sema 3A prevents OPC recruitment to demyelinated lesions and that Sema 3F can attract OPCs to lesions (Piaton et al., 2011). These data indicate that manipulation of semaphorins or their receptors may overcome impaired recruitment of OPCs to MS lesions. Studying OPC migration in the context of demyelination for drug discovery will, however, require more complex assays than those for oligodendrocyte differentiation.

Regeneration of myelin sheaths of optimal size

The recently published phenotypic chemical screens described above highlight the value of simple cell-based assays for identifying compounds that promote a singular process i.e. oligodendrocyte differentiation. While the rationale of promoting oligodendrocyte differentiation to enhance remyelination in MS has become the central focus of recent strategies, it remains to be seen to what extent the compounds and targets identified using this approach improve functional outcomes for patients. For example, whether health and function is truly restored to remyelinated axons in humans remains to be tested. Indeed, it is well known that even in areas of significant remyelination, in both MS and animal models, remyelination is imperfect, whereby the myelin sheaths formed during regeneration are shorter and thinner than normal (Blakemore, 1974; M. B. Bunge et al., 1961; Gledhill et al., 1973; Prineas & Connell, 1979). Given increasing evidence that precise regulation of the number, length and thickness of myelin sheaths along axons is likely to fine-tune neuronal circuit function (Baraban et al., 2016; Fields, 2015; Seidl, 2014), it is not unreasonable to suggest that regeneration of myelin sheaths of normal parameters is desirable (Filley & Fields, 2016; Liebetanz & Merkler, 2006). Indeed, it is possible that restoration of normal myelin sheaths during remyelination might be an important goal to consider with the aim of tackling cognitive impairments associated with MS. Given the additional trophic and metabolic support functions of myelin (Fünfschilling et al., 2012; Y. Lee et al., 2012b; Nave, 2010; Saab et al., 2013), it is also plausible that the restoration of sheaths of normal size may be required to reinstate these roles optimally, and perhaps to provide best protection to axons from prospective degeneration.

Whereas oligodendrocytes have an inherent capacity to differentiate *in vitro*, myelin sheath formation necessarily requires an axon, or an axon-like physical substrate (Bechler et al., 2015; S. Lee et al., 2012a; Lubetzki et al., 1993). Therefore, the previous screens that identified multiple pro-differentiating compounds did not assess myelination per se. Emerging data now indicate that a combination of the

physical size (diameter) of axons (Almeida, Czopka, French-Constant, & Lyons, 2011; Bechler et al., 2015; Goebbels et al., 2016; S. Lee et al., 2012a) and axonal signals (e.g. (Gibson et al., 2014; Koudelka et al., 2016)), cooperate to regulate myelin sheath number, length, and thickness along CNS axons (see (Klingseisen & Lyons, 2017) for review). Why myelin sheaths appear to be shorter and thinner during default remyelination remains unknown. However, the process of remyelination can in principle restore myelin sheaths of normal thickness and length to axons (Fyffe-Maricich, Schott, Karl, Krasno, & Miller, 2013; Münzel, Becker, Becker, & Williams, 2014; Powers et al., 2013; Xing et al., 2014), suggesting that identification of mechanisms that regulate these parameters could lead to strategies to optimally regenerate myelin. Although we are now starting to gain insight into the mechanisms that regulate myelin sheath growth, such studies are typically in-depth phenotypic analyses that are not readily suited for large-scale drug discovery. Therefore, robust and reductionist automated assays to study axon-myelin interactions with sufficient scale are required.

Additional opportunities to treat the progressive stages of MS

The enhancement of oligodendrocyte differentiation represents one opportunity to promote the regeneration of myelin for the treatment of MS. In addition to direct manipulation of the oligodendrocyte lineage, it is clear that axons, neural stem cells, microglia/ macrophages, Schwann cells, astrocytes, cells of the vasculature, as well as additional innate and adaptive immune cells, are also likely to represent possible therapeutic targets for the treatment of the progressive stages of MS. Consideration of the diverse pathologies of MS and potential strategies and entry-points for its treatment are beyond the scope of this review, but have been extensively described and reviewed elsewhere (Barnett & Lington, 2013; Correale & Farez, 2015; Dombrowski et al., 2017; Domingues, Portugal, Socodato, & Relvas, 2016; Franklin & Goldman, 2015; Liddel et al., 2017; Lloyd & Miron, 2016; Nait Oumesmar et al., 2008; Ransohoff, Hafler, & Lucchinetti, 2015a; Ransohoff, Schafer, Vincent, Blachère, & Bar-Or, 2015b; Waxman, 2008; Yuen et al., 2014; Zawadzka et al., 2010). In the final section of this review we focus on emerging technologies that will play important roles in dissecting the basic biology of disease and in providing platforms that allow screening for drug discovery.

Cutting edge technologies to understand glial function, cell-cell interactions, and drive drug discovery

The phenotypic cell-based assays discussed at the beginning of this review focus entirely on oligodendrocyte lineage cells in isolation. In doing so, they lack the fundamental physical substrate required for the process of myelination, axons, in addition to the dynamic communication that occurs between oligodendrocytes and their surrounding microenvironment. A number of current and emerging technologies

are providing new possibilities for studying cell-cell interactions *in vitro* using platforms that more closely resemble the *in vivo* environment. In the following sections, we will discuss the expanding field of microfluidics and how miniaturisation of liquid-delivery systems is improving the spatiotemporal control of drug delivery. We will then consider how this technology has been integrated with co-culture and organotypic slice culture preparations to study the processes of de- and remyelination *in vitro* before focusing on recent advancements in disease modelling and drug development using 3D organoids, and “organs-on-chip.”

Microfluidics

Microfluidics, or chip-based miniaturized fluid handling systems is a technology that has greatly expanded over the last couple of decades. Originally developed from fabrication technologies within the microelectronics industry, it has advanced into an interdisciplinary field that aims to model physiological microenvironments through the use of engineered platforms (Manz, Graber, & Widmer, 1990). At the heart of microfluidics is the ability to manipulate the flow of fluids within engineered microchannels. A variety of fabricated designs have been developed that enable multiple cell types to be either cultured within a single compartment or separated across multiple chambers inter-connected by microchannels, some of which have been applied to the co-culture cells of the nervous system (Neto et al., 2016).

Modelling myelination with microfluidics

Multi-compartment microfluidic devices composed of separate neuronal-soma and axon-glia compartments connected via microchannels have been fabricated to study myelination. In one of the first designs, neurons cultured in a central soma chamber extended axons along arrays of microchannels into separate glia compartments containing oligodendrocytes (J. Park, Koito, Li, & Han, 2009). Although this design only permitted a single treatment to be applied across the entire platform, more recent systems allow independent fluid control to separate neuron and glial compartments (Majumdar, Gao, Li, & Webb, 2011) or to multiple glial compartments in parallel enabling the effects of compounds on specific cell types to be studied (J. Park, Koito, Li, & Han, 2012). In these examples, cultured OPCs differentiated into mature OLs, as assessed by MBP expression, and were shown to form sheath-like structures resembling myelin around axons (Kerman et al., 2015; J. Park et al., 2012).

A challenge associated with the use of microfluidics in high-throughput myelination screening is accurately quantifying myelin sheath formation in response to chemical treatments (Kerman et al., 2015). Previous co-culture studies have relied on manual approaches to count or trace individual MBP+ segments of myelin following drug treatments (I. H. Yang et al., 2012). Apart from being incredibly time-consuming and limited to small-scale comparisons of chemical treatments, these approaches are also inherently subjective. Therefore, the development of computational approaches for the accurate, automated analysis of myelination are paramount. Zhang et al.

were the first to use a computational approach based on the overlapping fluorescence of myelin, visualised with MBP staining and axons, using Neurofilament staining, respectively, to quantify myelination per axonal area (H. Zhang, Jarjour, Boyd, & Williams, 2011). Using this as an index of myelination, they were able to show that treatment with factors reported to affect myelination and re-myelination *in vivo* had a similar effect in a cerebellar slice culture model (H. Zhang et al., 2011). Taking a similar fluorescence co-localisation approach, Kerman et al. reported a semi-automated method for myelin quantification in a microfluidic co-culture system that could be applied to large datasets (Kerman et al., 2015).

One interesting application of microfluidic co-culture devices could be for the study of neuronal activity-dependent regulation of myelination. In a report by Yang et al., applying intermittent electrical stimulation to DRG-glia microfluidic cultures (1hr/day) induced a five-fold increase in the number of myelin segments, confirmed by EM. This data suggests that the neuron-glia communication events that mediate activity-dependent myelination as reported *in vivo* may also be conserved *in vitro* and could allow for the careful dissection of neuronal versus oligodendrocyte components of this process (Gibson et al., 2014; Hines, Ravanelli, Schwindt, Scott, & Appel, 2015; Koudelka et al., 2016; Mensch et al., 2015; I. H. Yang et al., 2012). This finding was further exemplified in a recent study whereby optical stimulation of neurons expressing light-sensitive ion channels within microfluidic chambers resulted in increased numbers of myelin sheath structures (H. U. Lee et al., 2016). While vesicular-mediated signalling between neurons and oligodendrocytes has been reported (Frühbeis et al., 2013; Hines et al., 2015; Koudelka et al., 2016; Mensch et al., 2015; Wake, Lee, & Fields, 2011; Wake et al., 2015), the exact signalling events underlying activity-dependent regulation of myelination are still unknown. Therefore, the accessibility of microfluidic systems to biochemical analysis of isolated tissue (Taylor et al., 2005), electrophysiological recording (Biffi et al., 2012) and localised delivery of neuromodulators provide an exciting platform in which this process can be studied.

The small dimensions, low sample consumption, potential for automated analysis and parallelisation make microfluidic devices particularly attractive for high-throughput drug screening. While the number of reported microfluidic culture systems for investigating neuronal and/or glial drug responses is increasing, throughput still lags behind that of conventional multi-well cell cultures (H. S. Kim, Jeong, Koo, Han, & Park, 2016).

Organotypic slice cultures

Isolated living slices of brain tissue provide valuable *ex vivo* models given that they retain tissue architecture and circuit connectivity from their source of origin. In addition, with most of the major cell types present, the effect of exogenous treatments on the physiology of the tissue as a whole can be investigated. Major improvements in preservation have meant that organotypic slice cultures can be maintained for several weeks to months *in vitro* via perfusion with oxygenated

artificial cerebrospinal fluid, enabling long-term live imaging and electrophysiological recording (S. Cho, Wood, & Bowlby, 2007; Gogolla, Galimberti, DePaola, & Caroni, 2006). These advantages make organotypic slice cultures a useful model system in which to investigate responses to chemical treatments.

Studying de- and re-myelination in organotypic slice cultures

While *in vitro* co-culture studies of myelination provide great flexibility in terms of the genetic background of the cells used (e.g. combining mutant neurons with wildtype oligodendrocytes and vice versa), *ex vivo* organotypic slice cultures largely retain the cellular environment in which myelination occurs *in vivo* (for review see (Jarjour, Zhang, Bauer, Ffrench-Constant, & Williams, 2012)). Using viral-mediated gene delivery of fluorescent proteins to label oligodendrocytes within slice cultures, the structural dynamics and behaviour of individual oligodendrocyte lineage cells have been followed during their migration and maturation (Haber, Vautrin, Fry, & Murai, 2009). Over time, endogenous oligodendrocytes within these cultures form membrane ensheathments around axons which are both positive for the myelin marker mbp and form clear myelin sheaths (H. Zhang et al., 2011). This attribute of organotypic slices has been exploited to investigate the processes of de- and remyelination *in vitro*. Although a number of methods have been used to demyelinate axons within slice cultures, one of the most characterised is that of treatment with the gliotoxin lysophosphatidylcholine (LPC) (H. Zhang et al., 2011). In 2004, Birgbauer et al were the first to demonstrate remyelination of axons following LPC treatment in a cerebellar slice culture model (Birgbauer, Rao, & Webb, 2004). While this manipulation does not model the inflammatory pathology of MS, the proliferation of OPCs following demyelination and the production of thinner myelin with shorter internodes is characteristic of that seen *in vivo* (H. Zhang et al., 2011). In addition, many compounds shown to improve remyelination *in vivo*, have demonstrated similar effects following LPC-induced demyelination of cerebellar or spinal cord slice cultures validating these models for the characterisation of remyelination-enhancing therapeutics (e.g. (J. K. Huang et al., 2011; Mei et al., 2014; Najm et al., 2015)). The possibility to carry out focal demyelination in slice cultures may also allow *ex vivo* analyses of OPC recruitment to lesions, which may provide an important experimental platform to interrogate further mechanisms relevant to overcoming this bottleneck to regeneration.

While these experiments highlight the value of slice culture models for drug screening, the throughput of such systems is invariably low compared with that of cell-based assays. This is in part due to the practical limitations of the slice preparation in addition to the lack of standardised perfusion systems for the local and stable application of drugs to slices. For these reasons, slice culture models have mostly been used as secondary assays to pre-select promising therapeutics for further *in vivo* analysis. Recently, organotypic slice cultures have been integrated within microfluidic systems enabling greater control over drug delivery and the potential for increased scalability.

Combining microfluidics with organotypic slice cultures

In 2009, Berdichevsky et al, published one of the first compartmented culture devices that allowed organotypic slice cultures to be maintained within a microfluidic platform. In this system, individual slices cultured within separate, fluidically-isolated compartments extended axons along connecting microchannels to form synaptic connections with axons from the neighbouring slice. Using slice cultures from the entorhinal cortex and hippocampus, functional synapses were reported to form between the two brain regions enabling synchronization of network activity across the two-slice network. Additionally, selective pharmacological modulation to block spontaneous activity within one slice could be applied without affecting the activity of the other (Berdichevsky, Sabolek, Levine, Staley, & Yarmush, 2009). While this study demonstrated the potential of microfluidic slice culture systems for investigating circuit-specific effects following chemical treatments, it was limited in throughput to one chemical treatment per slice. Recently, a microfluidic system allowing multiple drug responses to be assessed in parallel was reported (T. C. Chang et al., 2014). Using a spatially-defined network of microchannels, multiple compounds were delivered simultaneously to pre-defined locations within a single mouse brain slice. Treatment with a concentration series of the cytotoxic agent STS demonstrated significant cell death selectively in STS-treated areas, which increased in a dose-dependent manner (T. C. Chang et al., 2014). Intended primarily for assaying chemotherapeutics, the benefits of this system could be applied to the cytotoxicity profiling of compounds, more generally providing a means by which small libraries of compounds could be assayed relatively quickly for unwanted side effects.

While these rodent-based models provide valuable model systems, the possibility of studying a patient's own tissue may give unprecedented insight into disease development and the effects of potential therapeutics, a possibility that is currently being realised with 3D organoid technology.

Studying the CNS microenvironment using 3D organoids

As previously highlighted, the complexity of the CNS can be defined not only by the diverse array of cell types and extensive communications that occurs between them but also by the highly organised arrangement of these cells within the 3D environment. A recent approach to mimic this environment *in vitro* through the use of 3D organoid cultures has created new possibilities to model CNS physiology and disease with remarkable fidelity (Shamir & Ewald, 2014). 3D organoids have been generated using a variety of different protocols, however, they generally rely either on external patterning growth factors to direct formation of specific brain regions or utilise self-patterning and self-organisation principles in an effort to mimic those used by the brain during embryonic development (Eiraku et al., 2011). Initially generated using rodent embryonic stem cells (Eiraku et al., 2008), recent advancements in human stem cell culture have enabled the generation of 3D organoids from pluripotent human embryonic stem cells, multipotent somatic stem cells and induced-

pluripotent stem cells (iPSCs) allowing expansion directly from patient tissue (Lancaster et al., 2013). In addition, strategies for re-programming iPSC cells into all major cell types of the nervous system including neurons (Hunsberger et al., 2015), oligodendrocytes (Prasad et al., 2016), astrocytes (Jiang et al., 2016; Zhou et al., 2016), and microglia (Muffat et al., 2016) have been developed providing an invaluable resource for studying human neurological disease. Using these techniques, protocols for the generation of human brain-like cerebral (Lancaster et al., 2013), cerebellar (Muguruma, Nishiyama, Kawakami, Hashimoto, & Sasai, 2015), forebrain (Qian et al., 2016) and mid-brain (Jo et al., 2016; Monzel et al., 2017) organoids have been reported.

3D organoids for disease modelling

A small number of studies have already begun to investigate the potential of patient iPSC-derived 3D organoid cultures to model disease (Lancaster et al., 2013; Raja et al., 2016) (Lancaster et al. 2013; Raja et al. 2016). Lancaster et al., showed that iPSC cells derived from a patient with severe microcephaly formed 3D cerebral organoids with smaller neural tissues and progenitor zones reflective of the reduced brain size of the patient (Lancaster et al., 2013). Similarly, iPSC cells generated from patients with familial AD were reported to recapitulate many AD-like pathologies, such as β -amyloid aggregation, hyper-phosphorylated tau protein and endosome abnormalities that are commonly missing from 2D cultures (Raja et al., 2016). One concern with using 3D organoid models is that their size may reduce compound penetration. However, in the latter study, treatment with β - and γ -secretase inhibitors was shown to reduce both the number of β -amyloid aggregates and level of pTau immunoreactivity suggesting that these model systems could be a potential avenue for drug testing (Raja et al., 2016). While many features of the human brain such as the 3D stereotypical cytoarchitecture and electrophysiological properties have been recapitulated in these models, at least in part (Lancaster et al., 2013; Monzel et al., 2017; Muguruma et al., 2015), the presence and function of glial cell types remains understudied. However, a recent study describing the development of a 3D midbrain-like organoid for the analysis of Parkinson's disease (PD), reported expression of S100 β and CNP-positive cells, indicative of astrocytes and oligodendrocytes within mature organoids (Monzel et al., 2017). In addition, 3D immuno-reconstructions revealed areas of CNP-positive staining that appeared to ensheath neurites suggesting that differentiated oligodendrocytes may functionally myelinate axons in 3D culture. Although further work will be required to fully characterise glial cell types within these models, if they do recapitulate cell function *in vivo*, 3D organoids may provide a means to investigate the mechanisms of myelin dysregulation using patient-derived cells. In addition, this creates new opportunities for phenotypic and mechanistic drug screening in complex human disease models.

While it is clear that the 3D complexity and relative ease with which 3D organoids can be generated represent significant advantages, do they represent useful models for high-throughput drug screening? In the following sections, we will suggest that

the recent integration of 3D organoids within miniaturised chips, termed 'organ-on-chip' technology has the potential to become a powerful tool for drug discovery and development.

Organs-on-chips

Integration of cutting edge innovations in microfluidics, 3D cell culture, vasculature modelling and micro sensors for electrophysiological and biochemical analyses are transforming the way in which we study basic physiology and drug development. These new *in vitro* models, termed "organs-on-chips" may not necessarily mimic the function of an entire organ, but rather recapitulate specific aspects of its function on a single mm-sized device. Through reproducing organ-specific 3D structure, microenvironment and physiology, a number of devices have been generated to mimic the lung, heart, kidney, liver, intestine and brain (Agarwal, Goss, Cho, McCain, & Parker, 2013; Choucha Snouber et al., 2013; Huh et al., 2010; H. J. Kim & Ingber, 2013; Schwartz et al., 2015). These platforms not only allow visualisation of dynamic, organ-level responses that cannot normally be observed in conventional 2D cell culture modes, but can also be used to facilitate drug development. Many hit compounds fail during clinical testing due to poor efficacy, unexpected adverse drug effects and poor blood brain barrier (BBB) permeability leading to costly withdrawals (Esch, Bahinski, & Huh, 2015). Therefore, improving the effectiveness of preclinical testing to better predict human drug responses is of critical importance.

Improving drug development with organs-on-chips

One recent use of organ-on-chip systems is in preclinical pharmacokinetic (PK) and pharmacodynamic (PD) modelling of drugs. Determining how much of a compound will reach its physiological target *in vivo*, and the final pharmacological effect is a vital stage in drug development and is normally carried out through mathematical modelling. However, for these models to be predictive, they require accurate *in vitro* data on drug metabolism and physiology (J. B. Lee & Sung, 2013). Conventional 2D cell cultures are unable to capture the dynamic complexity of human drug responses often resulting in model predictions that are wholly inaccurate when tested in humans. Through using organ-on-chip technology, whereby multiple fluidic compartments each holding a single biomimetic organ can be inter-connected, physiologically-relevant data on model multi-organ interactions can be obtained (J. B. Lee & Sung, 2013). In addition, organ-on-chip technology could reduce the need for whole animal testing. Generally, safety and toxicity testing are carried out in whole animals prior to clinical testing in humans. However, these experiments are slow, costly, require significant amounts of compounds and cross-species differences can often result in false positives. Therefore, using human-derived cells on 3D microplatforms may be one method by which the validity of toxicity testing can be improved, while minimising animal usage, cost and reagent volumes (Crofton et al., 2011).

Modelling the BBB with organ-on-chip technology

Dysfunction of the BBB is at the centre of a number of diseases including MS. An early event in relapsing-remitting forms of MS is hyperpermeability of the BBB resulting in increased migration of peripheral immune cells into the CNS which both increases inflammation and exacerbates BBB dysfunction (Larochelle, Alvarez, & Prat, 2011). Given the role of BBB dysfunction in MS, finding compounds that improve the integrity of the BBB or restrict migration of immune cells into the brain therefore represent important clinical targets. For example, Natalizumab (Tysabri), a humanized recombinant monoclonal antibody that inhibits leukocyte migration into the brain has been shown to reduce inflammation, occurrence of active lesions and relapse rates in relapsing-remitting MS (Hutchinson, 2007). In addition to directly targeting the BBB to prevent the entry of inflammatory cells or pathogens during disease, enabling the delivery of therapeutics to their sites of action within the brain is also critical. Many therapeutic compounds shown to improve functional outputs *in vitro*, often fail during clinical testing due to incomplete BBB penetrance. This highlights that the bottleneck in drug discovery is often not due to a lack of candidates but rather a lack of understanding of BBB physiology and suitable *in vitro* and *in vivo* tests of drug permeability. The reasons why many *in vitro* models fail to replicate the BBB faithfully *in vitro* is 1) they lack fluid flow and 2) they focus primarily on replicating the unique barrier function while neglecting the dynamic cell-to-cell communications that occurs between cells of the NGVU (Brown et al., 2015). By combining microfluidics with 3D multi-compartment cell culture, a number of BBB-on-chip devices have been generated that more closely mimic the NGVU environment (Achyuta et al., 2013; Brown et al., 2015).

In a study by Achyuta *et al.*, a multi-layered device was developed in which the top layer was populated by endothelial cells to mimic the vasculature, while a separate neural compartment containing neurons, astrocytes and microglia sat below separated by a porous membrane barrier. This layered set-up enabled the perfusion of fluid or pharmacological agents to be controlled separately to each compartment (Achyuta et al., 2013). Using this system, they were able to show that application of an inflammatory stimulus (TNF- α) to the vascular side of the device led to increased hyperpermeability of the endothelial barrier along with increased numbers of microglia and astrocytes displaying activated phenotypes as would be expected from *in vivo* data (Achyuta et al., 2013). This data indicates that models like this one may be useful for studying the influence of neuro-inflammation in disease. However, limitations of this assay include the use of rodent tissue rather than human, a lack of pericytes that would normally surround endothelial cells and a lack of fluid flow to mimic shear stress. An optimised model was recently reported that utilised human-derived cells to create separate neural and vascular chambers, which included cultured pericytes (Brown et al., 2015). Importantly, adjustable flow channels were used to perfuse fluid across endothelial cells to re-create the effect of shear stress *in vitro*. This resulted in a model system that mimicked many of the parameters of the BBB *in vivo* including expression of tight junction proteins, flow-orientated endothelial

cell polarity and functional passive and active transport mechanisms (Brown et al., 2015). The models discussed here could serve two important functions. Firstly, they could be used to study the physiology of the BBB under normal conditions and in CNS disorders where permeability is compromised, and secondly, they could be applied to the process of drug development as useful assays of drug permeability and toxicity.

While fabrication of these devices requires specialised equipment and expertise not available in most laboratories, companies are now developing cost-effective microfluidic platforms that are compatible with multi-well plate formats for cell culture and high-content screening (Tsui, Lee, Pun, Kim, & Kim, 2013; Volpatti & Yetisen, 2014). In addition, recent improvement in 3D cell culture is reducing tissue heterogeneity and increasing production efficiency enabling the development of standardised platforms for high-throughput compound screening, drug testing and disease modelling (Qian et al., 2016).

Whole-organism chemical screening

Although organ-on-chip technology has been heralded as the replacement to traditional *in vivo* models, whole animal testing is still the gold standard for drug discovery and development. Generally, this has involved the use of rodent models, but the low-throughput and high cost in terms of animals, time and money present significant drawbacks to their continued use. Small model organisms such as the zebrafish *Danio Rerio*, nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster* and African clawed frog *Xenopus* are providing attractive alternatives for drug screening due to their genetic amenability, low maintenance costs and fast development times. The advantages of such models for screening was realised early on and led to a number of influential large-scale genetic screens being carried out in *c. elegans*, *Drosophila* and zebrafish (Brenner, 1974; Nusslein-Volhard, 2012; Nusslein-Volhard & Wieschaus, 1980). More recently these model organisms have also been employed for chemical screening approaches (Strange, 2016). Unlike flies and worms, which do not have myelinated axons, the vertebrate zebrafish and *Xenopus* models share a number of attributes that make them particularly suited for the study of myelination *in vivo* (Bin & Lyons, 2016). To date, zebrafish have been employed for both chemical screens and studies of myelination to a greater extent than *Xenopus*.

Zebrafish

Over the last couple of decades, zebrafish have emerged as a powerful and versatile model organism for the study of developmental biology *in vivo* and, more recently, for small-molecule drug discovery. Their rapid external development and transparency during larval stages allows non-invasive live imaging of glial development and myelination (Czopka, Ffrench-Constant, & Lyons, 2013; Nawaz et al., 2015; Snaidero et al., 2014). Furthermore, discovery screens and targeted gene

analyses have revealed novel mechanisms of myelination and conservation with mammals (e.g. (Kazakova et al., 2006; Lyons, Naylor, Scholze, & Talbot, 2009; Monk et al., 2009; H. C. Park, Mehta, Richardson, & Appel, 2002; Pogoda et al., 2006; Snyder, Kearns, & Appel, 2012)). It could be argued that zebrafish are also uniquely suited for high-throughput chemical screening. Zebrafish larvae are only a few millimetres in length making them particularly amenable to treatment within multi-well plates and the ease with which large numbers of larvae can be generated (a single pair mating can produce several hundred offspring) provides a fast and expandable source of embryos. A key advantage of using zebrafish for chemical screening is their ability to absorb small molecules directly from the surrounding media making delivery of compounds relatively straightforward and flexible in terms of treatment dosage or timing. In this way, phenotypic changes in response to chemical treatments can be quickly and easily screened in the context of a whole, developing animal. Additionally, this quality endows zebrafish with a built-in means to assess drug specificity, efficacy and toxicity, parameters which are often hard or impossible to measure in cell-based assays and are often the reason why compounds fail during *in vivo* testing.

Chemical screens in zebrafish to identify therapeutics for MS

Since the first published zebrafish chemical screen in 2000, there have been over 65 small-molecule screens using zebrafish reported in the literature (R. T. Peterson, Link, Dowling, & Schreiber, 2000; Rennekamp & Peterson, 2015). During this period, advances in phenotypic characterisation, data collection and analyses have led to the identification of a number of potential therapeutic compounds, with a handful currently under investigation in clinical trials (Rennekamp & Peterson, 2015). The success of zebrafish as a model organism for drug discovery can in part be attributed to their high degree of homology to humans. Following completion of the zebrafish genome sequencing project in 2013, over 70% of human genes are now believed to have at least one obvious orthologue in zebrafish (Howe et al., 2013). This high degree of conservation is also evident in their pharmacology with an ever-increasing number of compounds tested in humans having similar effects in zebrafish (Milan, Peterson, Ruskin, Peterson, & MacRae, 2003; Rennekamp & Peterson, 2015). These similarities have made the zebrafish a popular model organism in which to study the processes of glial cell development and function.

Despite the clear advantages to using zebrafish to study glial development and myelination, few chemical screens have been published in this area. In 2010, Buckley *et al.*, published a drug reprofiling chemical screen in zebrafish to identify compounds with potential pro-myelinating properties (Buckley et al., 2010). Using the Tg(Olig2:GFP) transgenic line to identify alterations in oligodendrocyte lineage migration, proliferation and differentiation, treatment with a library of over 1000 compounds identified ~2% of compounds that altered oligodendrocyte number in the fish (Buckley et al., 2010). Secondary screening for alterations in myelination was carried out using real-time PCR for myelin basic protein, which identified three

compounds with specific effects, including a src family kinase inhibitor known to affect myelination in mammals (Osterhout, Wolven, Wolf, Resh, & Chao, 1999). Taking a complementary strategy to find therapeutic compounds for the inflammatory aspects of MS, Cusick et al., developed a screen to identify suppressors of T cell activation, a central feature in the immunopathogenesis of MS. Using a transgenic line to label T cells, Lenaldekar was identified as a novel inhibitor of activated T cell proliferation (Cusick, Libbey, Trede, Eckels, & Fujinami, 2012). In addition, treatment with Lenaldekar in an experimental autoimmune encephalomyelitis (EAE) rodent model of MS inhibited relapse severity, highlighting conservation in function across species (Cusick et al., 2012). In a similar vein, Vibsantin B, a novel macrocyclic diterpenoid was identified in a zebrafish chemical screen for inhibitors of leukocyte migration (Ye et al., 2015). Acting preferentially through heat shock protein (HSP)90 β , Vibsantin B was shown to ameliorate CNS inflammation and demyelination in EAE mice through inhibiting leukocyte infiltration into the CNS (Ye et al., 2015).

The availability of new reporters that allow assessment of myelinating cell number and extent of myelination (Almeida et al., 2011) directly *in vivo* will allow screens to be carried out with a greater focus on myelination per se. In addition, the variety of reporter lines labelling different populations of cells, such as neurons (Sato, Takahoko, & Okamoto, 2006), Schwann cells (Gilmour, Maischein, & Nusslein-Volhard, 2002), microglia/macrophages (Ellett, Pase, Hayman, Andrianopoulos, & Lieschke, 2011; Peri & Nusslein-Volhard, 2008; Sieger & Peri, 2013) and cells of the vasculature (Jin et al., 2007) expands the type and nature of chemical screens that can be carried out in zebrafish. Furthermore, the availability of zebrafish models of human disease generated by gene targeting or identification through gene discovery allows for chemical rescue screens and studies to be carried out that modify disease-related phenotypes. For example, it was recently shown that clemastine treatment could rescue hypomyelination in *mct8* mutant zebrafish (Zada, Tovin, Lerer-Goldshtein, & Appelbaum, 2016). In addition, as with humans and rodents, zebrafish possess a BBB which begins to form from just 3dpf in the larvae (Xie, Farage, Sugimoto, & Anand-Apte, 2010), allowing one to study the breakdown of barrier permeability, central to conditions such as MS, or to screen for therapeutic compounds capable of crossing the BBB *in vivo*. Given the promise of using zebrafish for chemical screening, significant effort is currently being invested in automating various aspects of the screening pipeline from mass embryo production systems, automated handling and positioning of embryos (T.-Y. Chang, Pardo-Martin, Allalou, Wählby, & Yanik, 2012; Pardo-Martin et al., 2013; 2010; Pulak, 2016), high-speed imaging, and automated analyses (G. Wang et al., 2015; White et al., 2016). Advances in these areas will help ensure that zebrafish also contribute to identifying and developing novel and disease-relevant therapeutics.

Conclusions

Glial cells are now known to regulate many aspects of nervous system formation, function and health. Their disruption is prevalent in arguably all diseases of the CNS, whether representing primary causation or secondary consequence of disease. In any case, manipulation of glia may represent therapeutic options for many disease states. Here we have focussed on the working model that promoting the endogenous capacity of our CNS to regenerate myelin may be an important means by which to treat MS. MS is a complex disease, and it is entirely likely that its life-long management will require multiple integrated strategies. Targeting the adaptive immune system will remain core to the treatment of the early stages of disease and possibly also later stages. However, we expect that this will be augmented by interventions that promote remyelination. The recent success of hypothesis driven studies and phenotypic screening studies indicate that medicines to enhance oligodendrocyte differentiation may soon be on the horizon. It is possible that they will be followed by interventions that target other cell types and cell-cell interactions to allow or enhance remyelination. Drug development focussed studies surrounding the roles of additional cell types and their interactions currently lag behind those aiming to promote oligodendrocyte differentiation, but are feasible. Here we also aimed to provide an overview of at least some technologies that could be employed to study cells of the nervous system and their interactions with therapeutic agents in more detail and at greater scale. Such approaches are obviously in no way restricted to analyses of remyelination and we anticipate that they will play key roles in the continued establishment of glial cells as generally relevant targets for the treatment of disease.

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	Target	Manipulation	Proposed effect on remyelination	Therapeutic translation
Identified from target driven strategies	Lingo 1	Anti-lingo1 antibody	Blocks signals inhibitory to differentiation	Phase II trial completed
	Nogo-A	Anti-Nogo-A antibody	Blocks signals inhibitory to differentiation	Second Phase I trial on-going
	Unknown	rHIgM22 antibody	Unknown	Phase I trial on-going
	RxR	Bexarotene (pan RXR agonist) Selective agonists in development	Promotes oligodendrocyte differentiation	Trial in recruitment phase
	Wnt pathway	Tankyrase Inhibitor (XAV939)	Blocking signals inhibitory to differentiation	Not yet pursued-affects many cell types
	Mitochondrial permeability transition pore	Olesoxime	Unknown	Phase Ib trial completed, well tolerated
	Phosphodiesterase-4	Inhibitor (Rolipram)	Unknown	Poorly tolerated in previous trials-affects many cell types
	Gpr17	Pranlukast (non-selective antagonist)	Promotes oligodendrocyte differentiation	Not yet pursued
	Endothelin receptor	PD142,893 (pan-antagonist) BQ3080 (EDNRB agonist)	Promotes oligodendrocyte differentiation and myelination	Not yet pursued
	Gli1	Gant61	Blocking signals inhibitory to OPC recruitment	Not yet pursued

Identified from hypothesis generating screens	Muscarinic acetylcholine receptor	Benztropine (Antagonist)	Promotes oligodendrocyte differentiation	Not yet pursued
		Clemastine (Antagonist)		Phase II trial completed
		Solifenacin (Antagonist)		Not yet pursued
	κ -opioid receptor	(\pm)-U-50488 (KOR agonist)	Promotes oligodendrocyte differentiation and survival	Not yet pursued
	Glucocorticoid receptor	Clobetasol (Agonist)	Promotes oligodendrocyte differentiation	Not yet pursued
Unknown acting on MAPK	Miconazole (Unknown)	Promotes oligodendrocyte differentiation and myelination	Not yet pursued	

Table 1. Summary of candidates and compounds that promote remyelination in vivo that have therapeutic potential

	Joubert et al. (2010)	Deshmukh et al. (2013)	Mei et al. (2014)	Mei et al. (2016)	Najm et al. (2015)	Peppard et al. (2015)	Porcu et al. (2015)	Lariosa-Willingham et al. (2016)
Library	In house, LOPAC and NINDS	Not specified	SelleckChem Known Bioactives	SelleckChem GPCR Library	NCC-I/II	Sanofi Collection Subset	Prestwick chemical library	NCC-I/II
Compounds	3000	~100000	1000	~250	727	~73000	1200	727
Concentration	3, 30 or 60µM	1.5µM	1µM	1µM	5µM	3µM	1, 10 & 25µM	2 of 10, 2, 0.4 or 0.08µM
Organism	Mouse	Rat	Rat	Rat	Mouse	Rat	Mouse	Rat
Cell Type	<i>Oli-neu</i> cell line	Primary OPCs	Primary OPCs	Primary OPCs	EpiSC- derived OPCs	CG-4 cell line	<i>Oli-neu</i> line expressing MRF	Primary unpassaged OPCs
Readout	Process length (actin staining)	% MBP+ve cells	Relative %MBP vs PDGFRA +ve rings surrounding micropillars		Process Length / MBP+ve cells	Morphological analysis	Mean MBP expression	MBP+ve signal / MBP+ve cells
Hits	10 in 3 classes	54 in 19 classes	18	15	14	22	23	26
Hit Confirmation	Repeat, EC50, Primary cell assay, MBP mRNA + Protein analysis	Not specified	Not specified	Not specified	Orthogonal assay, EC50, <i>ex vivo</i> slice cultures	Repeat EC50	EC50	EC50, additional OL markers
Hits followed up <i>in vivo</i>	N/A	Benztropine	Clemastine	(±)-U-50488	Clobetasol and Miconazole	N/A	N/A	N/A
<i>In vivo</i> validation	None	Cuprizone and EAE- induced demyelination	Focal demyelination	Focal demyelination	Focal and EAE- induced demyelination	None	None	None

Table 2. Summary of phenotypic screens that identified compounds promoting oligodendrocyte differentiation

Box 1 - *In vivo* models of demyelination:

There are several commonly used animal models of demyelination, each with their own advantages and disadvantages.

1. Experimental autoimmune encephalomyelitis (EAE):

- Animal is sensitised to spinal cord homogenate, myelin protein or peptide fragment
- T-Cell mediated demyelination
- Blood brain barrier compromised
- Many variants of model exist
- Demyelination is somewhat variable in localisation and extent and occurs concomitantly with axonal injury

2. Focal gliotoxin-induced demyelination:

- Chemical induced demyelination
- Several treatments used, including: ethidium bromide, calcium ionophores, anti-galactocerebroside antibodies and, most commonly, lysolecithin (lysophosphatidylcholine)
- High degree of spatial and temporal control of demyelination
- No T-cell mediated inflammation
- Single episode of demyelination allows study of timing of regenerative remyelination

3. Systemic demyelination using cuprizone:

- Global treatment with copper chelating agent
- Reversible following treatment cessation
- No T-cell mediated inflammation
- Demyelination largely studied in the corpus callosum
- Co-treatment with rapamycin gives more extensive and prolonged demyelination