Heinrich Heine Universität Düsseldorf

Phenotypic screening for pharmacological compounds promoting p57kip2's protein nuclear shuttling and myelin repair

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To my family

“ἐὰν μὴ ἔλπηται ἀνέλπιστον οὐκ ἐξευρήσει, ἀνεξερεύνητον ἐὸν καὶ ἄπορον”

Ἡράκλειτος, Αρχαίος ’Ελληνας Φιλόσοφος 544-486 π.Χ.

„If you do not expect the unexpected you will not find it, for it is not to be reached by search or trail”

Heraclitus, Ancient Greek Philosopher 544-486 BC

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1. SUMMARY/ ZUSSAMMENFASSUNG

1.1. Summary

Multiple sclerosis (MS) is one of the most widespread and debilitating demyelinating diseases, mainly affecting young people. An altered immune attack leads to oligodendrocyte, the myelinating cells of the central nervous system (CNS), and myelin loss and eventually to neuronal death and permanent neurological deficits. A population of resident oligodendroglial precursor cells (OPCs) remains an undifferentiated state throughout adulthood. In response to injury or disease, these cells can differentiate to myelinating oligodendrocytes, thus contributing to myelin repair. However, the innate remyelination capacity of the CNS is inefficient and declines with disease progression or ageing, due to the presence of inhibitory signals or the absence of stimulatory cues. The currently used therapeutic approaches for MS are mostly immunomodulatory and lack regenerative properties. There is nonetheless a plethora of studies focusing on remyelination promoting therapies with many of them, especially during the past decade, unraveling pharmacological approaches with these properties. As the inefficient capacity of adult OPCs to differentiate into mature oligodendrocytes is the rate-limiting factor for remyelination, most studies attempt to promote this process. Despite the large number of the already suggested pharmacological compounds and the fact that some of them have already entered clinical trials, sufficient remyelination in MS patients is still an unmet need and deciphering the molecular mechanisms regulating oligodendroglial cell development is going to pave the way for efficient regenerative treatments.

In this regard, previous work from our research group shed light to the early inhibitory role of the nuclear localization of the protein p57kip2. Although, nuclear exclusion of the protein is associated with OPC differentiation in a way that is dependent on binding to protein partners, the exact mechanism is not yet known. In this study, the subcellular localization of the p57kip2 protein was used as readout to establish a phenotypic screening for pharmacological compounds with the potential to promote transition from the OPC stage to mature myelinating oligodendrocytes. Out of the 21 preliminary hits that emerged from this

screening, four exerted differentiation promoting effects in rat primary OPCs. Danazol, a steroid drug, and the benzimidazoles parbendazole and methiazole, induced similar effects in human fetal OPCs and promoted developmental myelination in rat cerebellar organotypic cultures. Interestingly, danazol and parbendazole also promoted spontaneous OPC differentiation and remyelination in the cuprizone-induced mouse model of demyelination. In summary, this study suggests that early and evolutionary conserved cellular processes involved in the process of differentiation are applicable for identification of regeneration promoting treatments. Specifically, using a novel differentiation competence marker, a few substances were found to promote oligodendroglial cell development, while parbendazole and danazol are suggested as promising therapeutic candidates for demyelinating diseases, such as MS.

1.2. Zusammenfassung

Multiple Sklerose (MS) ist eine der häufigsten und belastendsten demyelinisierenden Krankheiten, die vor allem junge Menschen betrifft. Ein veränderter Immunangriff führt zum Verlust von Oligodendrozyten, den myelinisierenden Zellen des zentralen Nervensystems (ZNS), und von Myelin und schließlich zum Zelltod von Neuronen und zu dauerhaften neurologischen Defiziten. Eine Population von residenten oligodendroglialen Vorläuferzellen (engl.: oligodendroglial precursor cells, OPCs) bleibt im gesamten Erwachsenenalter in einem undifferenzierten Zustand. Als Reaktion auf Verletzungen oder Erkrankungen können sich diese Zellen zu myelinisierenden Oligodendrozyten differenzieren und so zur Myelinreparatur beitragen. Der Grad der angeborenen Remyelinisierungskapazität des ZNS ist jedoch insgesamt ineffizient und nimmt mit dem Fortschreiten der Erkrankung oder mit dem Alter ab, da hemmende Signale vorhanden sind oder stimulierende Reize fehlen. Die derzeit verwendeten therapeutischen Ansätze für MS sind meist immunmodulatorisch und haben keine regenerativen Eigenschaften. Dennoch gibt es eine Vielzahl von Studien, die sich auf remyelinisierungsfördernde Therapien konzentrieren, wobei viele von ihnen, insbesondere im letzten Jahrzehnt, pharmakologische Ansätze mit eben diesen Eigenschaften hervorgebracht haben. Da die Fähigkeit der adulten OPCs, sich in reife

Oligodendrozyten zu differenzieren, unzureichend und somit der limitierende Faktor für die Remyelinisierung ist, zielen die meisten Studien darauf ab, eben diesen Prozess zu fördern. Trotz der großen Anzahl dieser vorgeschlagenen pharmakologischen Substanzen und der Tatsache, dass einige von ihnen bereits in klinische Studien eingetreten sind, ist eine ausreichende Remyelinisierung bei MS-Patienten immer noch ein ungedeckter Bedarf, und die Entschlüsselung der molekularen Mechanismen, die die oligodendrogliale Zellentwicklung regulieren, wird den Weg für effiziente regenerative Behandlungen ebnen.

In diesem Zusammenhang haben frühere Arbeiten unserer Forschungsgruppe die frühe inhibitorische Rolle der nukleären Lokalisierung des Proteins p57kip2 beleuchtet. Obwohl der nukleäre Ausschluss des Proteins mit der OPC-Differenzierung insofern verbunden ist, dass er von der Bindung an Proteinpartner abhängt, ist der genaue Mechanismus noch nicht bekannt. In dieser Studie wurde die subzelluläre Lokalisation des p57kip2-Proteins als Indikator verwendet, um ein phänotypisches Screening nach pharmakologischen Substanzen zu etablieren, die das Potenzial haben, den Übergang von OPCs zu reifen myelinisierenden Oligodendrozyten zu fördern. Von den 21 vorläufigen Treffern, die aus diesem Screening hervorgingen, übten vier differenzierungsfördernde Effekte auf primäre OPCs der Ratte aus. Danazol, ein Steroid-Medikament, und die Benzimidazole Parbendazol und Methiazol induzierten ähnliche Effekte in menschlichen fetalen OPCs und förderten die entwicklungsbedingte Myelinisierung in organotypischen Kleinhirnkulturen von Ratten. Interessanterweise förderten Danazol und Parbendazol auch die spontane OPC-Differenzierung und Remyelinisierung im Cuprizon-induzierten Mausmodell der Demyelinisierung. Zusammenfassend konnte in dieser Studie gezeigt werden, dass frühe und evolutionär konservierte zelluläre Prozesse, die in den Prozess der Differenzierung involviert sind, für die Identifizierung regenerationsfördernder Substanzen geeignet sind. Mit einem neuartigen Differenzierungskompetenzmarker konnten einige Substanzen identifiziert werden, die die Entwicklung oligodendroglialer Zellen fördern, während Parbendazol und Danazol als potente therapeutische Kandidaten für demyelinisierende Erkrankungen wie MS vorgeschlagen werden.

2. INTRODUCTION

2.1. Cells of the nervous system

2.1.1. Neurons and glial cells in health and disease

Neurons and glial cells are the two major cell types of the vertebrate nervous system. Neuronal cells process and convey information via electrical and chemical signals (Rizo, 2018; Rutecki, 1992). While initially glial cells, also termed neuroglia, were considered to simply fill the space among neurons or to exert structural support, ideas originating from Rudolf Virchof, further work from descended neuroscientists like Santiago Ramon y Cajal and Pio del Rio-Hortega highlighted the cellular and functional properties of it, thus leading the way to an extended field of studies (Kettenmann & Verkhratsky, 2008). Nowadays we know that glial cells consist 50% of the cells in the human brain and they can reach up to 66.4% in other organisms (Azevedo et al., 2009; Herculano-Houzel, 2014). Neuroglia is divided into macroglia and microglia (Butt & Verkhratsky, 2018). The latter are cells of mesodermal/mesenchymal origin which constitute the innate immunity of the central nervous system (CNS), performing cell debris clearance both in development and adulthood, thus contributing to proper axonal development and survival, response to injury and disease and tissue repair (Ginhoux et al., 2013; Lima et al., 2010). Astrocytes along with oligodendrocytes, the myelinating cells of the CNS, are the major cell types belonging to macroglia, cells of neuroectodermal origin. Among their numerous functions, astrocytes are responsible for osmotic and neurotransmitter regulation, formation of the blood-brain-barrier (BBB), ion and energy homeostasis (Kimelberg, 2010; Kimelberg & Nedergaard, 2010; Verkhratsky & Nedergaard, 2018). Other cells of the CNS macroglia are ependymal cells, which line along the ventricular system and radial glia, multipotent progenitors that give rise both to neurons and astrocytes (Del Bigio, 1995; Howard et al., 2008). Only recently chondroitin sulfate proteoglycan NG2-positive cells were described as a distinct glial type of the CNS according to their diverse antigen repertoire (ffrench-Constant & Raff, 1986; Peters, 2004). NG2-glia belongs to the oligodendroglial cell lineage and keeps on giving rise to mature oligodendrocytes even throughout adulthood (Nishiyama et al., 2016). Schwann cells

and satellite cells are the macroglial types in the peripheral nervous system (PNS) that can be considered the counterparts of oligodendrocytes and astrocytes, respectively, in the periphery (Bhatheja & Field, 2006; Hanani, 2005).

Complex neuron-glial interactions are required for proper function of the nervous system. Pathologic conditions and ageing both in the CNS and the PNS can exert a direct or indirect negative impact on neurons (Conforti et al., 2014; Dugger & Dickson, 2017; Hughes, 2002; O'Shea et al., 2017; Whitesell, 2010), oligodendrocytes (will be discussed in chapter 2.3.1), astrocytes (Molofsky et al., 2012; Sofroniew & Vinters, 2010; Trujillo-Estrada et al., 2019), microglia (Colonna & Butovsky, 2017; Hickman et al., 2018; Norden & Godbout, 2013), and Schwann cells (Balakrishnan et al., 2020; Monk et al., 2015). Alterations in the functionality of each of these cell types induces perturbations in the neuron-glial network and the rest of the cell types are called to adapt their functions in order to correct the imbalance and re-establish homeostasis. The following chapters will focus on oligodendroglial cell development and functions, as well as the molecular and cellular signals that regulate their biology both in health and disease. This information is important for the understanding of oligodendrocyte related pathologies and the successful development of corresponding therapies.

2.1.2. Oligodendroglial cell characterization and origin

Oligodendrocytes produce myelin sheaths which then enwrap axonal segments, thus facilitating rapid action potential propagation and trophic support, while at the same time they preserve axonal integrity (Bunge, 1968; Dupree et al., 2004; Nave, 2010a, 2010b; Wilkins et al., 2001). Mature myelinating oligodendrocytes extent several processes, each of which repeatedly enwraps single axonal segments (internodes) in a concentric way to form a multispiral myelin sheath. The unmyelinated segments between the internodes are called nodes of Ranvier and each axon is being myelinated by several oligodendrocytes (Bunge et al., 1962; Bunge, 1968). The number of processes of a single oligodendrocyte which can myelinate varies from one to forty according to the location in the CNS and the species (Bjartmar et al., 1994; Bunge et al., 1961; Peters et al., 1991). Oligodendrocytes have been

assigned to four categories according to their size and the thickness of myelin they produce. The first category consists of the smallest cells which produce short and thin myelin and they can usually myelinate up to fifteen to thirty axons, while in the fourth category belong the largest cells which myelinate one to three axons with long and thick myelin sheaths (Butt et al., 1995). Oligodendroglial cell heterogeneity is also reflected by differences in their cytoplasmic densities and the degree of chromatin compaction. In this regard, electron microscopy enabled identification of light, medium and dark types of oligodendrocytes (Mori & Leblond, 1970).

Oligodendroglial precursor cells (OPCs) arise through sequential waves both in the vertebrate spinal cord and brain and they migrate until they reach their final destination, where they exit cell cycle and differentiate into mature oligodendrocytes [reviewed in (Bergles & Richardson, 2015)]. Cells positive for early oligodendroglial markers (OLIG2, Sox10, PDGFRa) are first detected in ventral ventricular germinal zones (VZ) of the spinal cord and brain. This event takes place around the embryonic day 12.5 (E12.5) in mice, E14 in rats and E45 in humans (Hajihosseini et al., 1996; Orentas & Miller, 1996; Pringle & Richardson, 1993; Timsit et al., 1995; Warf et al., 1991). During spinal cord development OPCs from the ventral neuroepithilium of the neural tube and specifically from the motor neuron progenitor domain (pMN) migrate throughout the spinal cord. Later, a second wave of OPCs derives from the dorsal spinal cord and migrates from the central canal to the pial surface (Cai et al., 2005; Fogarty et al., 2005; Tripathi et al., 2011; Vallstedt et al., 2005). Dorsally derived OPCs do not migrate as much as the ventrally derived ones. They populate the dorsal part of spinal cord to eventually replace their ventrally derived counterparts and they consist 20% of the total OPC population in the mouse spinal cord (Tripathi et al., 2011). A third wave occurs around birth and consists of expansion of the OPCs from the other waves and new ones from the central canal parenchyma, reviewed in (Rowitch & Kriegstein, 2010).

Similarly, in the forebrain, OPCs from the ventral VZ of the medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP) populate the entire developing telencephalon (embryonic developmental stage of the forebrain) including the cerebral cortex (Kessaris et al., 2006). This first wave is followed by a second dorsal one from the lateral and/or caudal ganglionic eminence (LGE and CGE) which moves dorsally to the cortex. A third wave arises within the early postnatal brain starting from the dorsal and outer subventricular zone (SVZ) to populate mainly the corpus callosum and in part the overlying cortex (Kessaris et al., 2006). Postnatally, ventrally derived OPCs are gradually eliminated in forebrain. The adult mouse brain is eventually mainly populated by OPCs derived from the SVZ and only 20% from the LGE (Tripathi et al., 2011).

The first wave of cerebellar OPCs stems from the metencephalic ventral rhombomere 1 region and migrates towards the cerebellum where it expands to populate the whole region, thus constituting the main pool of OPCs (Grimaldi et al., 2009; Hashimoto et al., 2016). A second local wave is generated around the cerebellar ventricular zone and it contributes only by 6% to the total population (Hashimoto et al., 2016). Finally, after birth neuroepithelial regions around the fourth ventricle may constitute source of cerebellar OPCs (Reynolds & Wilkin, 1988; Zhang & Goldman, 1996).

Specification of OPCs in the pMN progenitor domain is directed via the Sonic hedgehog (Shh) signaling from the notochord and the floor plate at the ventral midline which drives OLIG1 and OLIG2 transcription under the control of Nkx6.1 and Nkx6.2 (Agius et al., 2004; Cai et al., 2005; Lu et al., 2000; Orentas & Miller, 1996; Poncet et al., 1996; Vallstedt et al., 2005). The second wave of OPCs in the spinal cord is Shh independent and is regulated by the transcription factors Dbx1 and Ascl1, while reduction of bone morphogenetic proteins (BMPs) and regulation by fibroblast growth factor (FGF) signaling are also required (Fogarty et al., 2005; Mekki-Dauriac et al., 2002; Miller et al., 2004; Sugimori et al., 2008). The Notch signaling also collaborates with the Shh signaling for OPC specification (Kim et al., 2008; Park & Appel, 2003; Rabadán et al., 2012). In forebrain, Shh is expressed by precursor/stem

cells of the MGE and LGE to induce OPC production under the transcriptional control of Nkx2.1 and migration throughout the forebrain (Nery et al., 2001; Tekki-Kessaris et al., 2001). OPC specification of the second and third waves of production in the brain is regulated by the transcriptional factors Gsx2 and Emx1, respectively (Chapman et al., 2013; Kessaris et al., 2006). Wnt signals also influence OPC generation in the telencephalon (Langseth et al., 2010). In addition, collaboration between Shh and FGF signaling pathways is considered prerequisite for OPC specification in ventral telencephalon (Furusho et al., 2011; Kessaris et al., 2004).

2.1.3. Migration, proliferation and differentiation of oligodendroglial cells

Migrating OPCs are proliferative cells which are seeking for their final destination under the guidance of various extrinsic signals (Cohen, 2005; de Castro & Bribián, 2005; R. H. Miller et al., 1997; Pfeiffer et al., 1993). OPC migration is regulated by growth factors and morphogens like FGF-2, platelet-derived growth factor (PDGF-AA) or Shh and chemokines, like CXCL12, secreted by neurons and astrocytes. These chemotropic signals interact with molecules from the extracellular matrix (ECM) to exert motogenic effects. In addition, adhesion and contact molecules presented in the surfaces of neuronal axons and astrocytes, as well as in the ECM, interact with each other to regulate OPC adhesion. OPCs respond to extrinsic guidance according to their repertoire of receptors which are dynamically expressed during oligodendroglial development, reviewed in (de Castro et al., 2013). Migration and proliferation together constitute the recruitment of OPCs and as they are mainly concomitant events it is not a surprise that both processes share several similar regulatory mechanisms (Gard & Pfeiffer, 1990; Gard & Pfeiffer, 1993; Merchán et al., 2007). Neuronal activity is a crucial regulator of OPC proliferation (Barres & Raff, 1993). Most of the signals that promote OPC proliferation inhibit differentiation and support survival, thus fine-tuning oligodendroglial cell development (Baron et al., 2005; Canoll et al., 1996; Cui et al., 2010; Wang et al., 2007), reviewed in (Mitew et al., 2014). Once they reach their destination in the developing brain, OPCs expand to reach a homeostatic balance of their numbers and then they become less motile, exit the cell cycle and differentiate into mature oligodendrocytes which share similar

morphology, express myelin markers and have the potential to form myelin sheaths (Hughes et al., 2013), reviewed in (Baumann & Pham-Dinh, 2001). Cell cycle exit requires the cell cycle inhibitor p27kip1 and transcriptional repression of c-myc and e2f1 (Casaccia-Bonnefil et al., 1999; Larocque et al., 2005).

The oligodendroglial lineage is characterized by ubiquitously expressed markers with specific roles in the development, namely OLIG1 and 2, Sox10, Nkx2.2 (Emery, 2010). OLIG1 and OLIG2 are two related transcription factors with the first one playing a role in OPC differentiation in brain but not in spinal cord and the second one being essential for OPC specification (Dai et al., 2015; Rowitch, 2004; Xin et al., 2005; Zhou & Anderson, 2002). Sox10 is essential for OPC specification and differentiation and Nkx2.2 regulates the timing of differentiation (Kuhlbrodt et al., 1998; Pozniak et al., 2010; Qi et al., 2001; Stolt et al., 2004; Stolt et al., 2002; Zhu et al., 2014). Each stage of the oligodendroglial cell development can be defined by dynamic expression of numerous stage-specific markers and concomitant morphological changes [Fig. 1; reviewed in (Baumann & Pham-Dinh, 2001)]. Newly generated OPCs, are characterized by almost absolute absence of cell processes and expression of DM-20 mRNA, a splice variant of the proteolipid protein (PLP), the embryonic polysialylated form of neural cell adhesion molecule (NCAM), namely PSA-NCAM, PDGFRa and they still express the neuroepithelial stem cell marker nestin (Gallo & Armstrong, 1995; Grinspan & Franceschini, 1995; Lendahl et al., 1990; Pringle & Richardson, 1993; Timsit et al., 1995). Once committed to the oligodendroglial lineage, cells acquire a bipolar shape and do not express PSA-NCAM and nestin anymore, but surface antigens specific for the A2B5 antibody (Raff et al., 1984). These cells are called oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells because they not only give rise to oligodendrocytes, but also to astrocytes under specific conditions (Hardy & Reynolds, 1991; Hughes et al., 1988; Noble et al., 1988; Raff, 1989; Raff et al., 1983). Progenitor cells also express the gangliosides chondroitin sulfate proteoglycan type 4 (CSPG4 or NG2) and GD3 (Chang et al., 2000; Dawson et al., 2000; Hardy & Reynolds, 1991; Levine et al., 2001). Pre-oligodendrocytes extent more processes and are defined by additional expression of the surface lipid sulfatide, recognized

by the O4 antibody and 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (Braun et al., 1988; Sommer & Schachner, 1981). Maturing oligodendrocytes contact neuronal axons and express mature oligodendroglial markers including adenomatous polyposis coli protein (APC) clone CC1, glutathione-S-transferase pi cytoplasmic isoform (GST-pi) (Bhat et al., 1996; Tansey & Cammer, 1991) and myelin markers like the membrane marker galactocerebroside (GalC), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG), and myelin proteolipid protein (PLP) while they are becoming capable of producing functional myelin sheaths (Brunner et al., 1989; Linnington et al., 1984; Michalski et al., 2011; Raff et al., 1978; Timsit et al., 1995; Trapp, 1990).

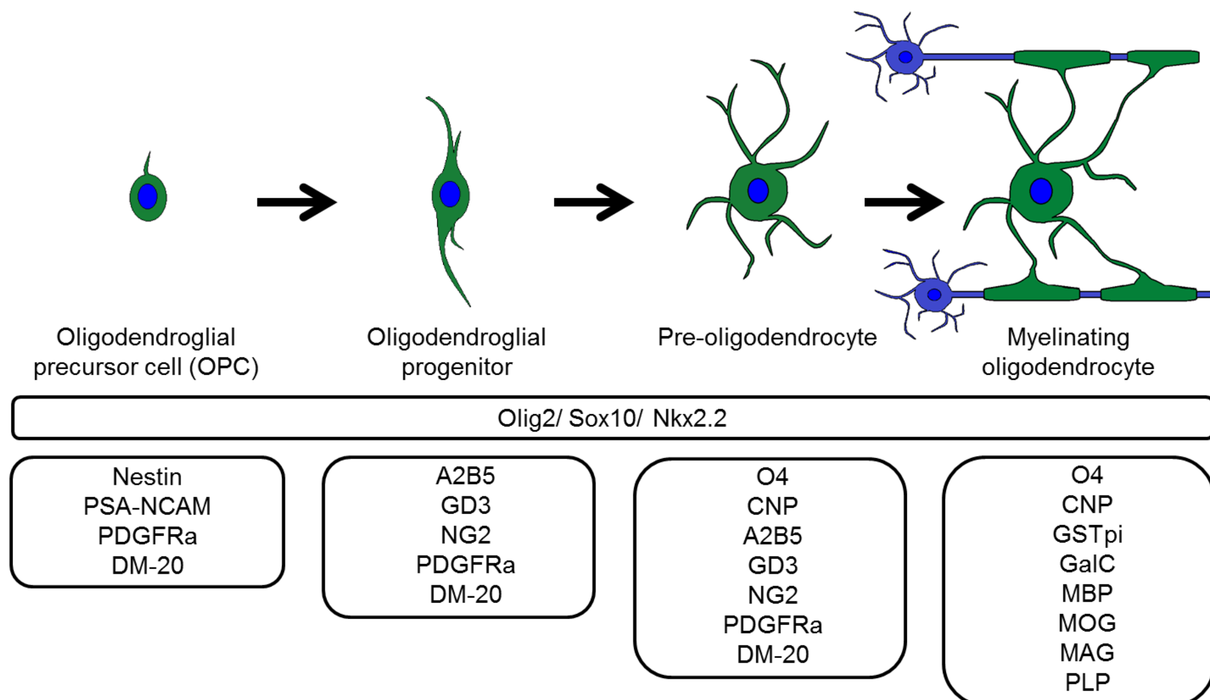


Figure 1. Schematic representation illustrating the developmental stages of the oligodendroglial lineage. Each stage is characterized by dynamic expression of oligodendroglial markers and gradual increase in morphological complexity.

2.1.4. Prominent markers of oligodendroglial cell differentiation

MBP is one of the most abundant membrane associated proteins representing 30% of the protein content in the CNS myelin and is expressed in numerous isoforms which are generated by alternative splicing (de Ferra et al., 1985; Nakajima et al., 1993; Zeller et al.,

1984). Deletion of the *mpb* gene, revealed implication of the protein in myelin compaction, a function which is most likely attributed to its positively charged residues and its interaction with the acidic lipids located in the opposing membrane (Cheifetz & Moscarello, 1985; Ding & Brunden, 1994; Roach et al., 1985). Low MBP expression levels are detected in immature cells, whereas these levels are increasing significantly during maturation, emphasizing its important role for the process of myelination (Baumann & Pham-Dinh, 2001; Dubois-Dalcq et al., 1986; Monge et al., 1986).

PLP is the most abundant myelin protein and contributes to physical stability of the myelin sheath via stabilization of the membrane junction upon myelin compaction (Boison et al., 1995; Klugmann et al., 1997). PLP and its isoform DM-20 are encoded by the same gene, formed by alternative splicing, and constitute 50% of the protein content in CNS myelin (Eng et al., 1968; Lees & Brostoff, 1984; Morello et al., 1986; Nave et al., 1987). Their expression starts early in development with DM-20 mRNA proceeding PLP, thus suggesting additional roles in migration and differentiation (Hudson, 2004). PLP interacts with molecules involved in vesicular transport and with integrins to modulate protein transfer in endoplasmic reticulum, abnormal protein elimination and interactions with the extracellular matrix (Gow & Lazzarini, 1996; Gudz et al., 2002; Yamaguchi et al., 1996). Ionophoric properties of PLP have also been reported (Cózar et al., 1987; Díaz et al., 1990).

APC (clone CC1) protein expression is detected in the cytoplasm of mature oligodendrocytes and regulates OPC differentiation in a Wnt/β-catenin signaling-dependent manner but also with additional β-catenin-independent mechanisms (Bhat et al., 1996; Lang et al., 2013). Significant indications suggest that APC regulates oligodendroglial cell differentiation via remodeling of the cytoskeleton (Lang et al., 2013; Näthke, 2006). APC expression is much higher in neurons than in mature oligodendrocytes and studies have shown that the CC1 antibody actually binds an epitope different than APC (Bhat et al., 1996; Brakeman et al., 1999; Lang et al., 2013). Recently was shown that the CC1 antibody, one of the most common tools to detect mature oligodendrocytes, binds a specific isoform of the RNA-

binding protein Quaking (QKI)7, which is enriched in myelinating oligodendrocytes (Bin et al., 2016).

Glutathione-S-transferases (GSTs) constitute a family of enzymes with many functions, including cellular detoxification. Cytosolic GSTs are assigned into five classes (alpha, mu, pi, sigma, theta), according to their structure and substrate specificity (Buetler & Eaton, 1992; Mannervik et al., 1985; Meyer et al., 1991). GST-pi isoform was initially described to be detected in the cytoplasm of mature oligodendrocytes and is a broadly used marker for this developmental stage (Tansey & Cammer, 1991). Analysis of the adult rat cerebral cortex showed that the intracellular localization of GST-pi can vary according to the differentiation state of the cell, with the nuclear localization being associated with progenitor stages and cytoplasmic with differentiated cells (Tamura et al., 2007).

The lysosomal galactocerebrosidase (GALC) enzyme catalyzes among others digestion of galactocerebroside, the major glycolipid in myelin, both in oligodendrocytes and Schwann cells (Kondo et al., 2005; Suzuki & Suzuki, 1970). Galactocerebroside (CG) has been described and is widely used as cell-surface marker for mature myelinating oligodendrocytes and Schwann cells (Cui et al., 2010; Raff et al., 1978; Ranscht et al., 1982).

2.2. Myelination

2.2.1. Myelin

The myelin sheath is an extension of myelinating cell plasma membrane that enwraps axonal segments in a concentric manner (Raine, 1984). Myelin sheaths wrap around axons forming the so-called internodes, in a form of alternating concentric layers consisting of compact and non-compact myelin [Fig. 2; (Bunge et al., 1962; Peters, 1964), reviewed in (Baumann & Pham-Dinh, 2001)]. Myelin membrane has a unique composition, low water content (40%), richness in lipids (70-85%) and low protein proportion (15-30%) that differentiates it from other biological membranes and ensures insulating properties, reviewed in (Rasband & Macklin, 2012). Cholesterol, phospholipids and galactolipids are the major lipids in CNS myelin and their content varies between 4:3:2 and 4:2:2, reviewed in (Rasband & Macklin,

2012). PLP and MBP, as mentioned above, are the main myelin proteins in the CNS, both exerting among others, stabilizing properties. PLP presumably forms “zipper-like” structures once myelin compaction is completed, while MBP locates in the cytoplasmic surface and stabilizes the major dense lines via dimer formation and interaction with negatively charged lipids (Boison et al., 1995; Klugmann et al., 1997; Privat et al., 1979; Roach et al., 1985), reviewed in (Rasband & Macklin, 2012). CNP is another prominent myelin protein which is concentrated in the cytoplasm of non-compact myelin but in close contact to the membrane surfaces and regulates extension and compaction of the myelin sheath (Barradas et al., 2000). MAG is also a non-compact myelin associated protein, localized specifically in the periaxonal myelin membranes, and is involved in adhesion and signaling between myelin-forming cells and axolemma (Li et al., 1994; Montag et al., 1994; Trapp & Quarles, 1982). MOG protein is localized on the outer surface of the sheaths and most likely participates in signal transduction (Taylor et al., 2004; Trapp et al., 1989).

Most of the internode is comprised of compact myelin which contains closely apposed plasma membranes and lacks cytoplasm. Internodal regions are visualized via electron microscopy as a series of alternating dark (major dense lines) and less-dark lines (intraperiod lines) which respectively represent the fused cytosolic protein layers of the cell membrane, emerged due to the lack of cytoplasm, and the outer protein layers, that are in close apposition but not fused with the neighboring membrane. Major dense lines and intraperiod lines are separated by unstained zones which correspond to lipid hydrocarbon chains, reviewed in (Rasband & Macklin, 2012).

Under the compact myelin are arranged the juxtaparanodal regions which are essential for the clustering of ion channels (Peles & Salzer, 2000; Rasband & Shrager, 2000). At the paranodal region, myelin is not compacted and contains cytoplasm as continuum of the cell body cytoplasm. At the lateral terminations of the internode, flanking cytoplasm containing membrane loops, called paranodal loops, form membrane complexes with the axonal membrane, the so-called transverse lines (Hirano & Dembitzer, 1982; Ichimura & Ellisman,

1991; Poliak & Peles, 2003). Another characteristic structure of the myelin sheath which contains cytoplasm is called Schmidt-Lantermann clefts. These clefts are located in the intermodal region and they are rare in the CNS but common in the periphery (Hall & Williams, 1970; Rasband & Macklin, 2012).

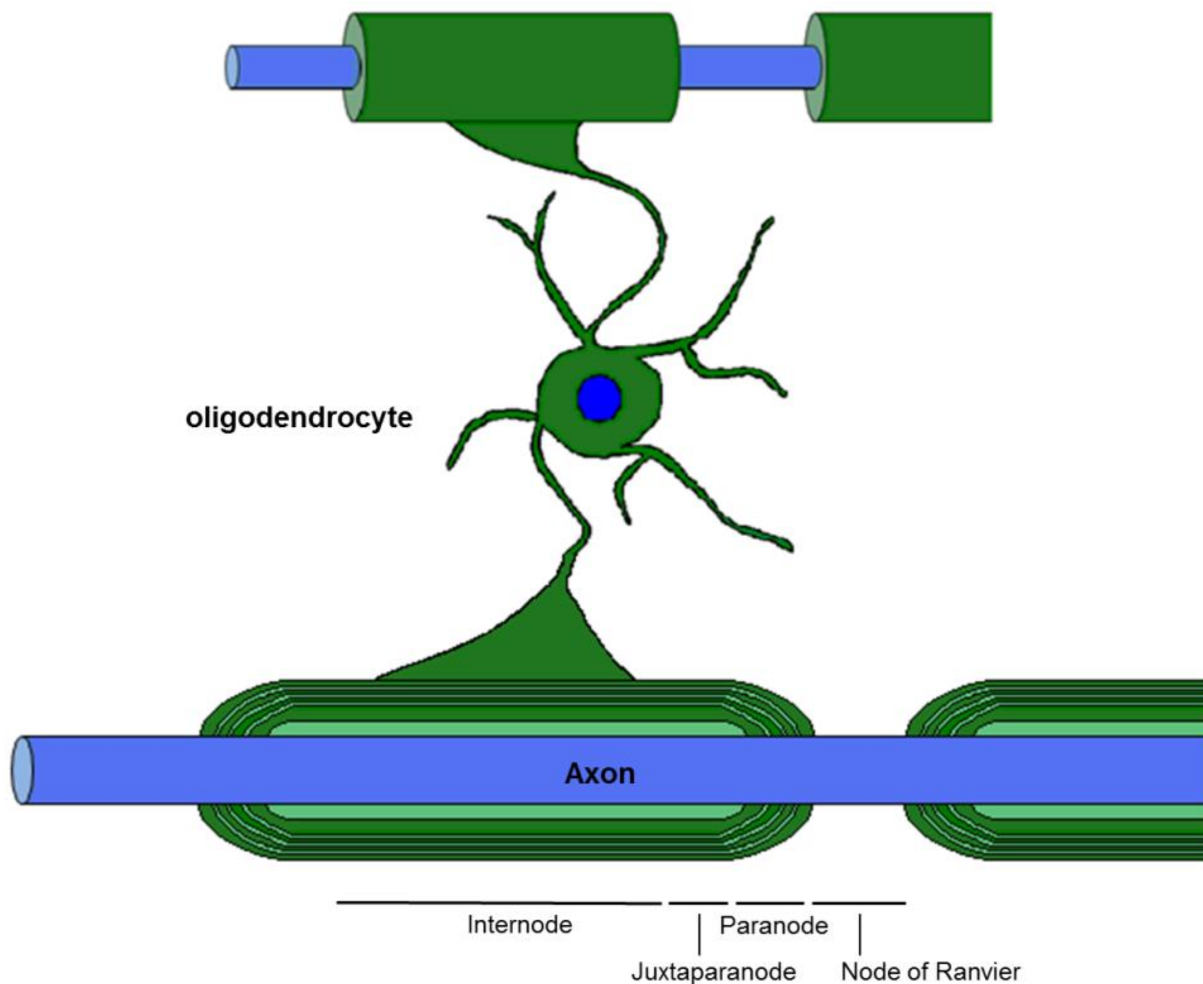


Figure 2. Simplified illustration of two axons myelinated by the same oligodendrocyte. Myelin sheaths enwrap axonal segments in a concentric way forming internodes, which are separated by unmyelinated segments, called the nodes of Ranvier. The central part of the internode consists of compact myelin which contains closely apposed plasma membranes and lacks cytoplasm. Laterally located structures called juxtaparanode and paranode regions consist of compacted and uncompacted myelin, respectively, and they play a role in ion channel clustering and oligodendrocyte-neuron interactions.

Along myelinated axons, adjacent internodes are separated by unmyelinated axonal segments, the nodes of Ranvier [Fig. 2; (Bunge, 1968)]. In these nodes a high density of voltage-gated Na^+ channels, Na^+/K^+ ATPase, Ca^{2+} ATPase and Na^+/Ca^{2+} exchangers are

clustered to mediate generation of action potentials, reviewed in (Waxman & Ritchie, 1993). Fast K^+ channels, responsible for re-excitation following an action potential are accumulated in the internodal and paranodal axolemma, underneath the myelin sheath, while slow K^+ channels, which modulate repetitive firing are located in the nodal axolemma, reviewed in (Waxman & Ritchie, 1993). Both via the insulating properties of its composition and the segmental formation which leads to clustering of the ion channels, myelin enables saltatory neuronal signal transduction. Electrical pulses are jumping from node to node, instead of progressing along the whole axon, thus increasing the conduction velocity 10 to 100-fold faster as compared to unmyelinated axons and reducing the energy demand (Hartline & Colman, 2007; Huxley & Stämpeli, 1949; Waxman, 1977, 1980).

2.2.2. Myelin formation

Several models have been proposed in attempt to explain how myelin sheaths are enwrapping axonal segments, reviewed in (Rosenbluth, 1999). The basis of the currently accepted model has been proposed already in 1954 and suggested that Schwann cell plasma membrane is extended as inner tongue, forms contact to the axon and expands laterally down to the axonal segment to form paranodal loops (Geren & Schmitt, 1954). Recently, combination of life cell imaging with 3D reconstruction led to the confirmation of this model. According to the model of “liquid croissant” and the work from Snaidero et al., upon conduct to the axon, oligodendroglial processes extend into a triangular shape and the inner tongue is wrapping around the axonal segment, while being in contact to the outer tongue, to form compact myelin. As soon as the myelin wrapping is sufficient, the cytoplasmic channels which allow communication between the inner and outer tongue are closing and myelination is terminated (Snaidero et al., 2014; Sobottka et al., 2012).

Myelination is a fine-tuned and restricted in terms of time and space process, which is regulated by several signals, reviewed in (Mitew et al., 2014; Nave & Werner, 2014). Axon ensheathment has four crucial steps a) selection of axon and initiation of cell-cell interactions b) establishment of stable contact and assembly of the nodes of Ranvier, c) regulation of

myelin thickness, d) longitudinal extension of myelin sheaths in response to axonal growth during postnatal life, reviewed in (Sherman & Brophy, 2005). Once differentiation has been completed oligodendrocytes have a narrow window of time to detect adjacent axonal segments and select which ones to myelinate. This time varies from five hours in the developmental zebrafish to 12 hours in myelinating co-cultures of rodent origin (Czopka et al., 2013; Watkins et al., 2008). *In vivo* time-lapse studies in zebrafish showed that oligodendrocytes dynamically extent and retract their processes towards adjacent neurons until they reach their final destination (Kirby et al., 2006). Sensing neighboring cells allows them to form myelin segments uniformly to ensure nodal regions of even length (Simons & Trotter, 2007).

Neuronal signals can negatively influence myelination, as for example the neuronal PSA-NCAM and LINGO-1 (leucine-rich repeat and Ig domain-containing, Nogo receptor-interacting protein), the latter of which upon binding to its NOGO receptor inhibits both myelination and process outgrowth (Decker et al., 2000; Fewou et al., 2007; Mi et al., 2005). On the other hand, neuronally produced laminin-a2 upon binding to β1 integrin receptors on oligodendrocytes promotes process extension (Colognato et al., 2002; Hu et al., 2009; Morissette & Carbonetto, 1995) and regulates myelin thickness, thus acting as positive regulator of myelination (Barros et al., 2009; Lee et al., 2006). Activation of the PI3K/Akt/mTOR (mammalian target of rapamycin) and Raf-MEK-ERK pathways act as additional positive regulatory signals for myelin thickness (Flores et al., 2008; Ishii et al., 2013). Myelination is also dependent on the density of OPCs (Rosenberg et al., 2008), oligodendrocyte Ca^{+2} concentration (Baraban et al., 2018), neuronal electrical activity (Gibson et al., 2014), astrocytic (Back et al., 2005; Hammond et al., 2014) and microglial/macrophage signals (Miron et al., 2013; Ruckh et al., 2012; Yuen et al., 2013). Although extrinsic signals, mostly derived from neuronal axons, tightly regulate myelin sheath formation, using microfibers Bechler et al. showed that oligodendrocytes can self-regulate formation of compact myelin and the length of the sheaths according to the axon caliber and their regional origin (Bechler et al., 2015).

2.3. Demyelination and remyelination

2.3.1. Demyelination

Demyelination is a pathological condition in which myelin sheaths covering axonal segments are lost either as consequence of an attack targeting oligodendrocytes, known as primary demyelination, or as secondary effect upon axonal injury or loss (Wallerian degeneration) (Qin et al., 2012; Traka et al., 2016; Valk & van der Knaap, 1989). Myelin destruction can be caused by several causes, including genetic mutations, toxicity, infections, or immune insult, reviewed in (Deber & Reynolds, 1991; Ludwin, 1997). Persistent demyelination leads to oligodendrocyte apoptosis, impaired saltatory nerve pulse conduction and prolonged refractory period, neurodegeneration and astrogliosis (Becher et al., 2006; Ferguson et al., 1997; Hemmer et al., 2002; Holley et al., 2003; Trapp et al., 1998). In response to demyelination, axons redistribute sodium channels along the axolemma resulting in excess of sodium influx into the axon. As demyelinated axons are bioenergetically challenged, their survival and the maintenance of the action potential are heavily dependent on the Na^+/K^+-ATPase. When ATP is not sufficient, calcium is accumulated and leads to activation of calcium dependent proteases and degeneration (Waxman, 2006). In general, imbalance between energy demand and generation of ATP leads to axonal loss both in acute and chronic demyelination, reviewed in (Mahad et al., 2015; Trapp & Stys, 2009). Increased mitochondrial activity in neurons is an innate neuroprotective mechanism in response to demyelination (Licht-Mayer et al., 2020; Mahad et al., 2015; Mahad et al., 2009; Mutsaers & Carroll, 1998). Despite the innate neuroprotective mechanisms, due to the deep axonal dependence to myelin in terms of preservation of integrity and trophic support and the importance of saltatory way of nerve conduction, normal conduction velocity, electrophysiological functions and energetic balance can only be restored via re-establishment of the myelin sheath (Nave, 2010a, 2010b).

As mentioned, myelin loss can be caused by a significant number of heterogeneous pathological conditions, reviewed in (Bercury & Macklin, 2015; Kremer et al., 2016a; Love, 2006). Inflammatory diseases such as multiple sclerosis (MS), reviewed in (Höftberger &

Lassmann, 2018), leukodystropathies including Pelizaeus-Merzbacher disease (Garbern, 2007), reviewed in (Bonkowsky et al., 2010), psychiatric disorders like schizophrenia (Flynn et al., 2003; Tkachev et al., 2003), reviewed in (Haroutunian et al., 2014; Nave & Ehrenreich, 2014), developmental disorders including attention deficit hyperactivity disorder (ADHD) (Wu et al., 2014) and Down syndrome (Fenoll et al., 2017; Reiche et al., 2021), and neurodegenerative disorders like Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS) (Defrancesco et al., 2012; Kim et al., 2013; Philips et al., 2013) have been associated with oligodendrocyte dysfunctions and demyelination.

2.3.2. Remyelination

Although CNS is not considered to acquire much regenerative potential, upon oligodendrocyte injury and demyelination a naturally occurring process, called remyelination, takes place leading to re-establishment of myelin sheaths and subsequent enwrapping of the demyelinated axons (Franklin & ffrench-Constant, 2008, 2017). Remyelination prevents neurodegeneration, sustains metabolic support, and restores rapid nerve conduction (Duncan et al., 2009; Franklin & ffrench-Constant, 2017; Irvine & Blakemore, 2008; Kornek et al., 2000; Mei et al., 2016b; Smith et al., 1979; Smith & Koles, 1970). The latter is dependent both on the thickness of the newly formed myelin sheath and re-arrangement of sodium channels in the node of Ranvier, which takes place during remyelination even earlier than the myelin repair [Fig. 3; (Coman et al., 2005; Smith & Koles, 1970)].

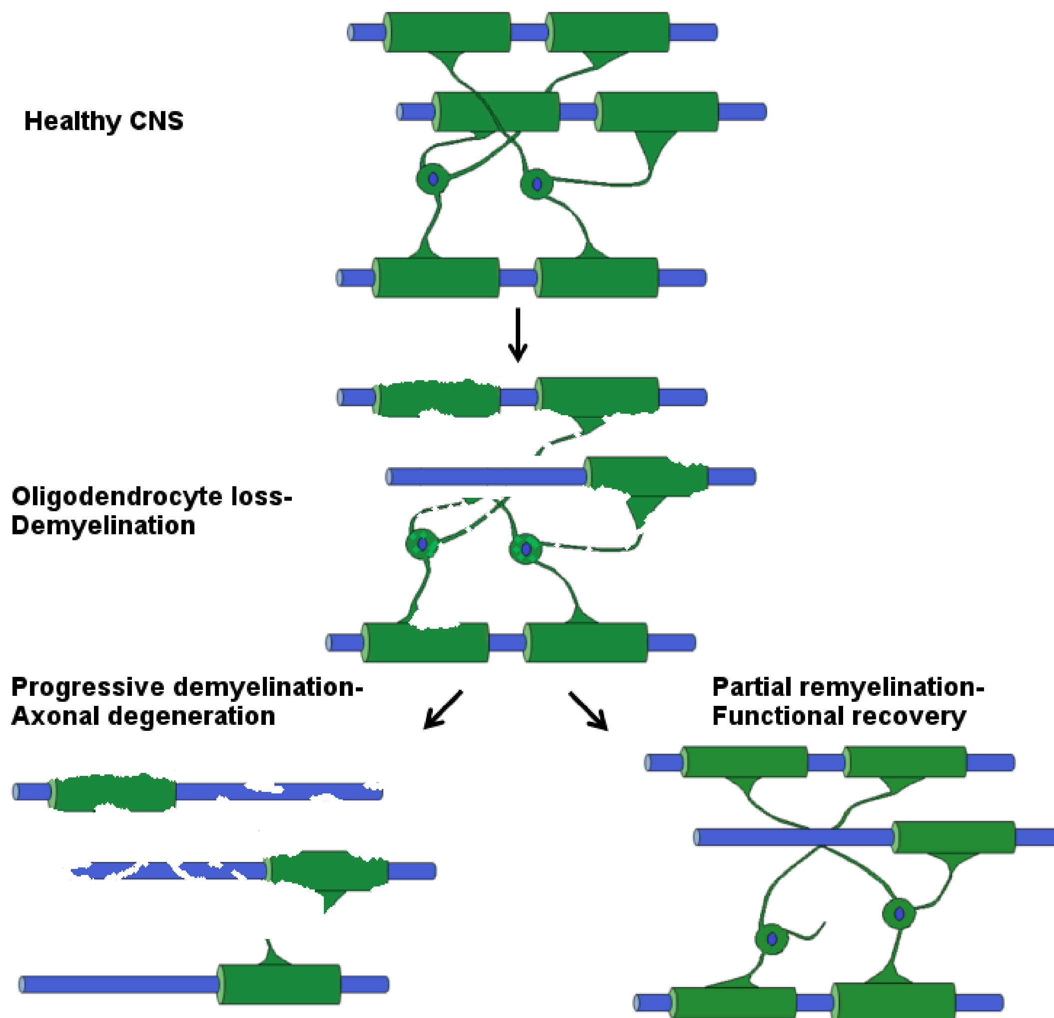


Figure 3. Schematic illustration of demyelination and remyelination in the CNS. Myelin sheaths covering axonal segments can be destroyed either as consequence of a direct oligodendrocyte attack (primary demyelination) or as secondary effect upon axonal injury or loss (not shown here). Without myelin repair activities, a progressive oligodendrocyte and neuronal loss takes place, leading to permanent neurological deficits. The innate CNS regeneration capacity can partially support re-establishment of the myelin sheath, a process called remyelination, thus leading to neuronal survival and functional recovery.

Remyelination in the CNS is mainly mediated by a resident NG2-positive oligodendroglial projenitor cell population which remains an undifferentiated state throughout adulthood and contributes to myelin maintenance and repair in case of disease or injury (Dawson et al., 2003; Nishiyama et al., 2016; Nishiyama et al., 2009). Often in the literature these cells are equated with precursor cells, therefore for simplicity reasons they will be referred to as OPCs throughout this thesis. Adult OPCs are distributed widely throughout both white and gray matter, comprise approximately 5% of the cells in the CNS and are characterized by slower

motility, poorer survival, and a prolonged cell cycle in comparison to developmental OPCs (Duncan et al., 2009). NG2-positive cells are mostly committed to the oligodendroglial cell lineage, thus contributing to re-establishment of the myelin sheath, however they have been associated with generation of astrocytes and neurons, as well (Kang et al., 2010; Kondo & Raff, 2000; Rivers et al., 2008; Zhu et al., 2008). Interestingly, it is also known that adult OPCs can give rise even to Schwann cells which can contribute to CNS myelin repair (Zawadzka et al., 2010). The determination of their fate is not yet well understood, therefore more delicate studies are required for this purposed, reviewed in (Nishiyama et al., 2016).

Remyelination, like myelination, is a fine-tuned process with distinguishable phases which are regulated by a plethora of factors, reviewed in (Franklin & ffrench-Constant, 2017; Taveggia et al., 2010). Adult OPCs are dispersed in the brain and they extend filopodia to detect perturbations, each of them conveying surveillance of its own territory (Hughes et al., 2013). Once they are activated, in response to injury, the recruitment phase initiates. Specifically, they re-enter the cell cycle to rapidly proliferate and then migrate to populate the site of the lesion where they eventually differentiate to myelinating oligodendrocytes (Kang et al., 2010; Moyon et al., 2015; Simon et al., 2011). Glial cells of the injured region secrete migration promoting and mitogenic signals, which induce differential gene expression in OPCs that leads to their activation (Fancy et al., 2004; Watanabe et al., 2004). Chemokine and cytokine release stimulate proliferation and migration, as well as differentiation and initiation of myelination (Arnett et al., 2001; Fok-Seang et al., 1998; Maysami et al., 2006; Vela et al., 2002; Zhang et al., 2006). Moreover, microglia/macrophage-mediated myelin debris clearance along with immune cell- and astrocyte-mediated growth factor release, positively affect OPC differentiation and remyelination (Döring et al., 2015; Kotter et al., 2006).

Whether pre-existing mature oligodendrocytes can also contribute to myelin repair is not yet clear, however for years it was thought that this is not the case, a notion based on genetic fate mapping studies which showed that these cells are post-mitotic, not motile and they fail

to remyelinate upon transplantation into lesion (Crawford et al., 2016; Targett et al., 1996). Moreover, it was shown that although pre-existing oligodendrocytes re-extent processes towards the axons upon injury, functional ensheathment does not take place (Keirstead & Blakemore, 1997). This notion was recently brought into question first by Duncan et al. who showed connections between pre-existing oligodendrocytes both with myelinated and remyelinated axons, using rodent and non-human primate models (Duncan et al., 2018). One year later Yeung et al., provided more evidence supporting those findings via measurements of ^{14}C integration levels in the genomic DNA (Yeung et al., 2019).

Despite the regenerative potential of the CNS, neurodegeneration can be detected despite remyelination, which is a phenomenon that intensifies with age (Hampton et al., 2012; Irvine & Blakemore, 2006; Lindner et al., 2009; Shields et al., 2000; Zhao et al., 2006). In addition, myelin sheath thickness upon remyelination is always thinner compared to sheaths emerged during myelination and according to computational studies the velocity of nerve pulse conduction cannot completely reach the normal levels (Gupta et al., 2004; Smith & Koles, 1970). Several studies addressing every phase of remyelination have attempted to explain the reasons for its failure and the relation to ageing. Regarding OPC availability, there are no available data indicating that adult OPC density declines with ageing (Doucette et al., 2010; Sim et al., 2002). In addition, it was shown that increasing the OPC numbers in focal demyelination mouse models does not enhance the remyelination capacity (Woodruff et al., 2004). Insufficient OPC migration into demyelinated sites was shown to cause delayed remyelination both in MS (see next chapter) and mouse models (Boyd et al., 2013). Changes in the lesion environment, as for example an increase in secreted signals from reactive astrocytes, can inhibit OPC migration (Omari et al., 2006; Sobel, 2005). In fact, it was postulated that astrocytes in the lesion impede the rate of remyelination via inhibition of process extension and migration (Fok-Seang et al., 1998). However, there are other studies which suggest that the presence of astrocytes in the lesion is necessary for remyelination to take place, and that they secrete factors which positively regulate this process (Ishibashi et al., 2006; Talbott et al., 2005). OPC activation is also regulated by the rate of inflammation

and myelin breakdown in the lesion (Chari et al., 2006; Franklin & Kotter, 2008). Moreover, recently was shown that proliferation is a prerequisite for differentiation of adult OPCs (Foerster et al., 2020). Finally, also factors that influence the myelin sheath thickness and length have attracted the scientific interest and studies have been developed towards the optimization of the process (Baraban et al., 2016; Lee et al., 2013; Mitew et al., 2014). All these studies prove that every stage of remyelination is dependent on regulatory mechanisms and can become a potential therapeutic target. However, the most prevailed postulation is that adult OPC differentiation is the rate-limiting phase of the process and the most potent target for the development of myelin repair treatments. This suggestion is supported both by studies using agents that promote OPC differentiation with recurrent enhancement of remyelination and decline in neurological impairment in aged mice and the finding that inflammatory demyelinated lesions in MS patients possess sufficient numbers of OPCs which are likely arrested in an undifferentiated state (Chang et al., 2002; J. Huang et al., 2011; Kuhlmann et al., 2008; Wang et al., 2020; Wolswijk, 1998).

2.3.3. Multiple sclerosis: a prominent demyelinating disease

Multiple sclerosis is the most commonly occurring autoimmune demyelinating disease of the CNS, causing neurological deficits associated with both motoric and cognitive dysfunctions (Noseworthy, 1999; Weiner, 2004). Mostly affected are young adults aged between twenty- and forty-years and the ratio of incidence between men and women is 1:2 (Koch-Henriksen & Sørensen, 2010; Kurtzke, 2000). The etiology of MS is complex and still not completely understood; however, prevalence and migration data clearly show that environmental influences play a role (Kurtzke, 2000; Pugliatti et al., 2002; Rosati, 2001). Viral infections have also been suggested to trigger MS development with Epstein-Barr virus and human herpesvirus 6 to be the most consistently implicated ones (Álvarez-Lafuente et al., 2004; Wagner et al., 2004). In addition, there is growing evidence of implication of human endogenous retroviruses of the HERV-W family both in MS and oligodendroglial cell development (Göttle et al., 2019; Kremer et al., 2019a; Kremer et al., 2013; Morris et al., 2019). Viral attacks likely act as MS co-factors either via an induction of an immune

response, which targets myelin, thus exposing myelin antigens, or via molecular mimicry between viral and myelin antigens (Virtanen & Jacobson, 2012). Genetic factors lead to a higher incidence among siblings and especially identical twins, while the major histocompatibility complex gene on chromosome 6p21 and the human leucocyte-antigen-DR2 allele have been suggested to be implicated (Barcellos & Thomson, 2003; The Multiple Sclerosis Genetics et al., 2002).

Most commonly, initial MS development is characterized by alternating relapses and phases of recovery, a disease form called relapsing-remitting MS (RRMS). Approximately 60-70% of those patients will develop later during the disease course secondary progressive MS (SPMS), which constitutes a progressive worsening of the symptoms. About 10% of the MS patients develop a similar disease course already during early stages, namely primary progressive MS (PPMS) (Dutta & Trapp, 2014; Hauser & Oksenberg, 2006; Trapp & Nave, 2008).

Tissue injury in MS is caused by an altered immune response, which initially is characterized by activation of autoreactive $CD4^+$ T cells in the periphery and their migration to the CNS (Frohman et al., 2006), reviewed in (Lassmann, 2014). Release of pro-inflammatory cytokines by activated lymphocytes leads to activation of infiltrated cytotoxic $CD8^+$ cells, production of antibodies and activation of microglia and macrophages, all of which target oligodendrocytes and myelin proteins (Coleman, 2005; Lassmann et al., 2007; Lucchinetti et al., 2000; Neumann et al., 2002; O'Loughlin et al., 2018; Trapp & Stys, 2009). As disease progresses, mechanisms like mitochondrial injury amplify oxidative stress (Murphy, 2009). Concomitant progressive axonal degeneration and oligodendrocyte loss lead to further activation of microglia, which exhibits cytotoxicity upon exposure to pro-inflammatory cytokines (O'Loughlin et al., 2018). Eventually, inflammation leads to activation of astrocytes and subsequent decrease in toxin clearance from the ECM, loss of trophic support to oligodendrocytes and neurons and additional production of pro-inflammatory cytokines in the CNS parenchyma (Ponath et al., 2018). As result, MS lesions are characterized by

breakdown of the blood-brain-barrier, inflammation, glial scar formation, oligodendrocyte apoptosis, demyelination and axonal degeneration (Trapp & Nave, 2008; Trapp & Stys, 2009).

Current MS therapies aim to prevent or suppress the autoimmune attacks via immunomodulation; therefore, they have no regenerative properties which could reverse already existed neurological deficits (Kremer et al., 2019b; Kremer et al., 2019c). CNS has innate capacity of myelin repair during initial stages of the disease; however, this regeneration potential is not sufficient, differs among MS patients and declines with the disease course due to inhibitory signals and absence of stimulatory cues, thus neurodegeneration and irreversible neurological damage remain inevitable (Franklin & ffrench-Constant, 2017; Gruchot et al., 2019; Kotter et al., 2011; Kremer et al., 2011; Kremer et al., 2016a; Louapre et al., 2015). Given that endogenous myelin repair by means of cell replacement takes place via recruitment, activation and differentiation of resident OPCs of the adult CNS (Chang et al., 2000; Franklin & ffrench-Constant, 2017), investigation of intrinsic and extrinsic factors that regulate these processes may lead to the development of myelin repair interventions which can be applied in MS therapy. As previously discussed, among the distinct phases of remyelination, differentiation of adult OPCs is considered to be the rate-limiting one especially in MS lesions (Chang et al., 2002; Kuhlmann et al., 2008; Wolswijk, 1998). Therefore, understanding the underlying regulatory mechanisms of this process may be the most promising approach for the development of successful myelin repair promoting treatments for MS.

2.3.4. *Ex vivo* and *in vivo* experimental models for the study of demyelination and remyelination

There is a number of available *ex vivo* and *in vivo* experimental models for the study of demyelination and remyelination, with some of them including activation of the immune system as means of mimicking the pathology of MS and others only addressing the pathophysiology of demyelination per se, reviewed in (Denic et al., 2011; Lassmann & Bradl,

2017; Merrill, 2009; Procaccini et al., 2015; Ransohoff, 2012; Torre-Fuentes et al., 2020). The currently used *in vivo* models are toxin-mediated, auto-immune and inflammatory, viral induced, genetic or zebrafish models. Until recently *ex vivo* demyelination models were only induced by means of toxin application, while recently another approach was developed to recapitulate disease pathologies (Liu, 2019).

In 2004, an *ex vivo* lysolecithin (lysophosphatidylcholine, LPC)-mediated model of demyelination using cerebellar slices from postnatal day 10 (P10) rats was established (Birgbauer et al., 2004). Lysolecithin, a bioactive lipid, induces focal demyelination of white matter tracts when injected into areas such as the cerebellar peduncle and spinal cord (Hall, 1972; Plemel et al., 2018; Woodruff & Franklin, 1999). This model has been proven effective for the identification of remyelination-promoting properties of chemical compounds (Eleuteri et al., 2017; Miron et al., 2010). Recently, IgG1 monoclonal recombinant antibodies from clonally expanded cerebrospinal fluid (CSF) plasmablasts from MS and neuromyelitis optica (NMO) patients were applied on cerebellar organotypic slices (Liu, 2019). These disease-specific antibodies were used for induction of a complement-dependent cytotoxicity that initiates mimicking of the disease pathologies. This is the first approach to induct immune mediated demyelination *ex vivo* for the study of demyelination and remyelination.

Cuprizone is a copper chelator that causes mitochondrial dysfunction selectively in mature oligodendrocytes leading to apoptosis and eventually demyelination which can mainly be detected in corpus callosum, anterior commissure, thalamus and cerebellar peduncles (Matsushima & Morell, 2001). Upon dietary treatment of mice with cuprizone, demyelination takes place with little or no implication of adaptive immunity and upon retrieval of the treatment spontaneous remyelination initiates, thus making this model one of the first choices when it comes to testing remyelination capacity of chemical compounds and ideal to investigate molecular and cellular aspects of de- and remyelination (Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Kipp et al., 2009; Suo et al., 2019; Zhu et al., 2019) reviewed in (Kipp et al., 2009; Skripuletz et al., 2011; Vega-Riquer et al., 2019). Cuprizone-

mediated lesions resemble those of pattern III MS and demyelination and remyelination in part coincide similar to MS pathology (Liu et al., 2010). A modification of this model consists of additional rapamycin injections that induce more extensive demyelination and prolonged remyelination period, characteristics which help the evaluation of the impact of remyelination treatments (Chen et al., 2017; Narayanan et al., 2009; Sachs et al., 2014). Another approach for induction of focal demyelination involves microinjections with LPC or ethidium bromide, with the latter one being less used nowadays (Blakemore et al., 1977). These two chemicals cause lesion formation in areas such as the hippocampus, striatum, optic nerve and spinal cord (van der Star, 2012). LPC damages directly the myelin sheath but also causes inflammatory response via chemoattraction of monocytes (Jeffery & Blakemore, 1995). The effect of ethidium bromide is based on intercalation with DNA mainly in astrocytes and is mediated via a reduction in the trophic support by those cells (Lassmann & Bradl, 2017; Merrill, 2009).

Experimental autoimmune encephalomyelitis (EAE) is an inducible, T-helper cell-mediated rodent model for MS which is used as tool to investigate pathophysiological aspects of the disease and therapeutic interventions, including regenerative therapies. The main targets of the immune response are proteins produced by myelinating oligodendrocytes and the primary results of the immune attack are demyelination of axonal tracts, impaired neuronal signal transduction and motoric symptoms, thus making this model suitable for the assessment of the impact of pharmacological treatments both based on their remyelination promoting and immunomodulatory properties, reviewed in (Denic et al., 2011; Robinson et al., 2014). However, the occurrence of the lesion development and the initiation of the regenerative processes cannot be well defined in this model; therefore, it is not suggested for exclusive study of remyelination (Ransohoff, 2012). Even though EAE application in primates results in pathology closer to MS in the human system than rodent models, the latter ones are more broadly used due to ethical and economic reasons (Kap et al., 2016; Torre-Fuentes et al., 2020). EAE can be induced directly by immunization with myelin proteins or peptides (active EAE) or via transfer of myelin specific CD4+ T lymphocytes to naïve animals (passive

EAE), with each approach recapitulating different characteristics of the disease progression, reviewed in (Robinson et al., 2014). In mice, EAE is actively induced by an immune response triggered by injection of immunogens derived from the myelin proteins PLP, MOG and MBP along with an adjuvant (Haanstra et al., 2013; Ransohoff, 2012; Yasuda et al., 1975). Immunization of SJL/J mice with the epitope $PLP_{139–151}$ induces relapsing–remitting EAE (Tuohy et al., 1989), while immunization of C57BL6/J mice with the $MOG_{35–55}$ peptide is associated with a relative controlled immune response and chronic progressive demyelination (Procaccini et al., 2015; Tompkins et al., 2002). While mice EAE models are the most prominent, the pathology has been successfully induced also in guinea pigs, zebrafish and rats, with the latter one manifesting inflammatory infiltration of mononuclear cells in cerebellum, spinal cord and medulla oblongata (Gambi et al., 1989; Kulkarni et al., 2017; Robinson et al., 2014).

Demyelination in the CNS can be induced by viruses such as the picornavirus Theiler’s murine encephalomyelitis virus (TMEV) and the coronavirus mouse hepatitis virus (MHV), reviewed in (Procaccini et al., 2015; Ransohoff, 2012; Torre-Fuentes et al., 2020). In these cases, demyelination is a result of an inflammation that is clinically manifested as encephalitis. TMEV induces an acute inflammation which is followed by a chronic, progressive stage of spinal cord demyelination and remyelination where oligodendrocytes, macrophages, microglia and astrocytes are affected (DePaula-Silva et al., 2017; McCarthy et al., 2012; S. Miller et al., 1997; Rivera-Quiñones et al., 1998; Tsunoda et al., 2007). Like in MS, demyelination occurs in sites where activated microglia and macrophages are accumulated (Lassmann & Bradl, 2017). MHV-mediated inflammation has a well-defined biphasic progression and can be used as an additional model for the study of MS pathology (Bergmann et al., 2006).

Zebrafish is a small, simple organism, with short embryonic development and a relatively well studied mechanism of myelin formation, reviewed in (D'Rozario et al., 2017; Preston & Macklin, 2015). For these reasons zebrafish is well-established in the study of

oligodendroglial cell differentiation, myelination and remyelination *in vivo*, reviewed in (Ackerman & Monk, 2016; Czopka, 2016). Transgenic zebrafish lines expressing green fluorescent protein have contributed both to the study of remyelination and the identification of compounds promoting myelin repair (Buckley et al., 2008; Buckley et al., 2010; Diamantopoulou et al., 2019; Early et al., 2018; Gong et al., 2001; Preston et al., 2019; Udvadia & Linney, 2003).

Finally, transgenic mice have also contributed to the study of myelin damage and repair. In order to investigate the role of myelin proteins both during myelination and remyelination, mouse models with duplication and inversion of the MBP gene (Shiverer) or PLP gene mutations (Rumpshaker and Jimpy), which lead to myelin damage, have been developed, reviewed in (van der Star, 2012). A few transgenic mouse models have been either used to induce or to alter the progression of EAE Pathology, reviewed in (Procaccini et al., 2015). Moreover, oligodendrocyte death can be induced by means of genetic manipulation. Specifically, in this approach, first genetic engineering allows insertion of a specific gene (e.g. diphtheria toxin A receptor) in the organism which can be conditionally expressed only in oligodendrocytes, and following administration of a chemical compound (such as diphtheria toxin A) to induce oligodendrocyte specific cell death (Pohl et al., 2011; Traka et al., 2010).

2.4. Intrinsic and extrinsic regulators of oligodendroglial cell differentiation

2.4.1. Extrinsic signals from other cell types

Neuronal activity regulates both oligodendroglial cell development and myelination, reviewed in (Baydyuk et al., 2020; de Faria et al., 2019). Although this regulatory role merely affects migration, proliferation, and myelination in an axon-specific manner, OPC differentiation is also affected (Barres & Raff, 1993; Gibson et al., 2014; Mitew et al., 2018). Studies based on optogenetic stimulation, pharmacogenomic activation and introduction of a complex motor learning task indicate that the increase of the number of mature oligodendrocytes in response to neuronal activity can be a result of both increased OPC proliferation and

differentiation but also a direct outcome of potentiated differentiation of newly formed OPCs without prior cell division (Gibson et al., 2014; McKenzie et al., 2014; Mitew et al., 2018; Xiao et al., 2016).

OPCs respond to neuronal activity via synaptic inputs, they express voltage-gated ion channels and several neurotransmitter receptors, reviewed in (Marinelli et al., 2016). In fact, voltage-gated Ca^{++} entry was shown to promote OPC maturation, voltage-gated K^{+} channels mostly regulate proliferation and cell cycle progression, thus fine-tuning the initiation of differentiation, and while the role of Na^{+} channels in oligodendroglial cell development is not clear yet, their expression decreases as OPCs mature (Cheli et al., 2015; Ghiani et al., 1999a; Paez et al., 2009).

Neuronal activity and glutamate release promote OPC differentiation and myelin repair via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors (Gautier et al., 2015). Activation of specific N-methyl-d-aspartate (NMDA) receptor subunits in OPCs regulates differentiation divergently, as revealed by the upregulation of the NMDAR1 and NMDAR2A and downregulation of NMDAR2B and NMDAR3 protein levels during the transition from OPCs to mature oligodendrocytes (Cavaliere et al., 2012; Sawada et al., 1996). OPCs also express functional adenosine receptors, which are activated upon action potential firing to stimulate differentiation and myelin formation (Stevens et al., 2002). In addition, ATP liberated from axons indirectly regulates OPC development, via triggering leukemia inhibitory factor (LIF) release from astrocytes, a molecule that promotes OPC maturation (Ishibashi et al., 2006; Mayer et al., 1994). Gamma-Aminobutyric acid (GABA), acting via the GABAA receptors, was shown to positively regulate cerebellar NG2-positive cell proliferation and differentiation (Zonouzi et al., 2015). The oligodendroglial lineage expresses serotonin (5-hydroxytryptamine, 5-HT) receptor subtypes 5-HT1A and 5-HT2A and 5-HT exposure was shown to adversely affect oligodendroglial cell development via aberrant outgrowth of cell processes and reduced myelin protein expression (Fan et al., 2015). Regarding catecholaminergic signals, differentiating oligodendrocytes express the

dopamine receptors D1 and D3 and it has been suggested that especially the receptor D3 plays a role in differentiation and myelin formation (Bongarzone et al., 1998; Rosin et al., 2005). Moreover, there are indications that β-adrenergic receptor activation stimulates the process of differentiation as well (Ghiani et al., 1999b). Rat OPCs express all types of muscarinic cholinergic receptors, with the M3 type being the most abundant, while mature oligodendrocytes express only moderate levels of M2, suggesting a regulatory role of cholinergic signals during cell maturation (De Angelis et al., 2012). Muscarine treatment was shown to alter myelin marker expression, hence suggesting a role in terminal differentiation, while acetylcholine release from neurons along with pharmacological interventions suggest that M3 antagonism could promote OPC differentiation and myelin repair (Abiraman et al., 2015; De Angelis et al., 2012; Goldman et al., 2012; Maruyama et al., 2008). Finally, modest levels of nitric oxide (NO), a gaseous neurotransmitter, promotes oligodendroglial cell growth and maturation and coordinates myelination, while high concentrations induce oxidative/nitrosative stress and uncontrolled MBP phosphorylation (Atkins & Sweatt, 1999; Garthwaite et al., 2015).

Astrocyte-derived factors tightly regulate oligodendroglial cell development, reviewed in (Baydyuk et al., 2020). As mentioned above, release of the factor LIF from astrocytes regulates the timing of oligodendrocyte development and myelination via promotion of cell maturation (Ishibashi et al., 2006; Ishibashi et al., 2009). Astrocyte-released PDGF inhibits OPC maturation, while FGF exerts the opposite effect, however, when these two factors act together, they keep cells in an immature and proliferative state (Bögler et al., 1990). OPCs express receptors for another soluble astrocyte-released signal called endothelin-1, which was shown to reduce OPC differentiation in the postnatal brain (Gadea et al., 2009; Yuen et al., 2013). Finally, astrocytes also secrete ECM proteins such as laminin and fibronectin, which affect oligodendroglial cell development, as will be discussed later, reviewed in (Kiray et al., 2016).

Macrophages and microglia support oligodendroglial cell development after injury via myelin clearance, which is a prerequisite for the initiation of differentiation, reviewed in (Miron & Franklin, 2014). Factors secreted by anti-inflammatory/immunoregulatory (M2) type microglia drive oligodendrocyte differentiation as seen by conditioned media application on oligodendrocytes *in vitro* or by M2 microglia depletion *in vivo* experiments (Miron et al., 2013; Pang et al., 2013). These factors are mainly cytokines which are either secreted directly from microglial cells or through astrocytes. Such examples are the interleukins IL-1b and IL-6 and the tumor necrosis factor a (TNFa), which act through astrocytes and LIF secretion, thus enhancing OPC differentiation (Fischer et al., 2014; Vela et al., 2002). Finally, in MS lesions and in EAE the cytokine CXCL12 is upregulated in endothelial cells, activated microglia and activated astrocytes. CXCL12 which is synthesized also in the meninges and by specific neurons under physiological conditions, was shown to promote spontaneous OPC differentiation *in vitro* via its CXCR7 receptor and *in vivo* during remyelination via the CXCR4 receptor, reviewed in (Chu et al., 2017).

2.4.2. Regulatory role of the extracellular matrix

ECM in the CNS is composed of hyaluronan (HA), chondroitin sulfate proteoglycans (CSPGs) and proteins such as laminin and fibronectin. Regulation and homeostasis of the expression of these molecules are essential for the timely transition of OPCs into mature oligododendrocytes in development and for myelin repair, while they are perturbed in the injured CNS and during ageing (Lau et al., 2013; Marangon et al., 2020; Segel et al., 2019; Srivastava et al., 2020; Wang et al., 2018; You & Gupta, 2018). HA interacts with the TLR2/4 and CD44 receptors, which are predominantly expressed by astrocytes and OPCs and acts as inhibitor of OPC differentiation as seen upon its accumulation in the injured white matter or upon conditional overexpression of CD44 (Su et al., 2017; Tuohy et al., 2004; Vinukonda et al., 2016). Selective pharmacological hyaluronidase inhibition was shown to effectively promote OPC maturation and remyelination *in vivo* (Preston et al., 2013; Su et al., 2020). CSPGs are expressed by all neural cell types, their function depends on their core proteins and they include four lecticans, namely aggrecan, versican, brevican and neurocan, the

RPTP/phosphacan and neuron-glial antigen-2 (NG2) (Dyck & Karimi-Abdolrezaee, 2015; Pu et al., 2018). CSPGs impair OPC differentiation and process extension (Karus et al., 2016; Pendleton et al., 2013), while reduction of their production or neutralization of their effects enhances oligodendroglial progression both in development and remyelination (Keough et al., 2016; Kuboyama et al., 2017; Stephenson et al., 2019). Fibronectin aggregation and fibroblast-secreted fibronectin were demonstrated to also inhibit OPC differentiation in LPC-induced demyelination model and EAE respectively, prompting the development of myelin repair therapeutic approaches which target this molecule (Qin et al., 2017; Stoffels et al., 2013; Wang et al., 2018; Yahn et al., 2020). On the other hand, laminin-2 is a positive regulator of OPC maturation, an effect which is exerted via interactions with the oligodendroglial receptor α6β1 and activation of the tyrosine-protein kinase Fyn (Buttery & ffrench-Constant, 1999; Lourenço & Grãos, 2016; Ly et al., 2018).

2.4.3. Other secreted regulatory signals

BMPs are secreted ECM-associated proteins and negative regulators of OPC differentiation, an effect mediated via an increase in the expression of the transcription factor Id4 and decrease in the expression of the transcription factors OLIG1 and 2, all of which will be discussed later, as well as via inhibition of interactions between Id4 and the OLIG proteins (Cheng et al., 2007; Samanta & Kessler, 2004). BMP2 and BMP4 were shown to inhibit oligodendrocyte maturation *in vitro* and at early stages they even shifted the cell fate towards the astrocytic lineage, while at the same time BMP levels below a critical threshold are considered prerequisite for the initiation of OPC differentiation during development (Gomes et al., 2003; Grinspan et al., 2000; Mabie et al., 1997; Miller et al., 2004; See et al., 2004). Increased BMP levels are associated with spinal cord injury, demyelination and MS, and hypoxic/ischemic injury and interestingly, BMP inhibition in experimental models of these pathologies led to recovery via an increase of oligodendrocyte maturation, reviewed in (Wheeler & Fuss, 2016).

The PSA-NCAM is another negative regulator of OPC differentiation and myelination, the down-regulation of which is required for the initiation of both processes (Decker et al., 2002; Fewou et al., 2007; Trotter et al., 1989).

LINGO-1 is expressed both in neurons and oligodendrocytes and negatively regulates OPC differentiation and (re)myelination (Lee et al., 2007; Mi et al., 2007; Mi et al., 2005). During development LINGO-1 expression is down-regulated at the onset of OPC differentiation, while during adulthood the injured CNS is characterized by higher levels of the protein (Mi et al., 2004; Mohan et al., 2014; Satoh et al., 2007). Lingo-1 mediates its effects via a tripartite formation with the Nogo-66 receptor (NgR1) and either the p75 neurotrophin receptor or the TAJ/TROY receptor (Mi et al., 2004; Park et al., 2005; Shao et al., 2005). Additionally, Lingo-1 was found to block the translocation and activation of a positive regulator of differentiation, called ErbB2 (Lee et al., 2014).

Autotaxin (ATX) and insulin-like growth factor (IGF-1) are on the other hand two secreted positive regulators of OPC maturation with the first one inducing changes in morphological and gene expression level, reviewed in (Wheeler & Fuss, 2016) and the second one via activation of the ERK1/2 kinases which will be discussed later (Hsieh et al., 2004; Shi et al., 2014; Ye et al., 1995).

Semaphorins are a family of secreted and transmembrane proteins which were found to be expressed by cells of the oligodendroglial lineage along with their receptors (Cohen et al., 2003; Zhang et al., 2014). Several members of this family were shown to regulate different stages of oligodendroglial development, reviewed in (Wheeler & Fuss, 2016) and regarding OPC differentiation Sema6A has been identified as a positive regulator, while Sema3A as a negative one (Bernard et al., 2012; Syed et al., 2011).

Sphingosine-1-Phosphate (S1P) is a lipid signaling molecule which exerts its functions upon extracellular release via five G protein-coupled S1P receptors, named S1P1–S1P5 (Maceyka et al., 2012; Proia & Hla, 2015). Oligodendrocytes express the subtypes S1P1, S1P2, S1P3

and S1P5 (Novgorodov et al., 2007; Yu et al., 2004). A structural analog of S1P, termed FTY720-phosphate (Fingolimod) is a potent agonist for S1P1, S1P3, S1P4, and S1P5 (Zemann et al., 2006). Low concentrations of Fingolimod exert cytoprotective effects and promote OPC differentiation (Coelho et al., 2007; Cui et al., 2014; Jung et al., 2007), while higher concentrations induce negative effects (Coelho et al., 2007; Jung et al., 2007; Miron et al., 2008; Novgorodov et al., 2007). Fingolimod failed to induce remyelination in toxin-mediated *in vivo* models of demyelination, when in contrary increased remyelination in organotypic cerebellar slices (Hu et al., 2011; Kim et al., 2011; Miron et al., 2010). Light in the exact role of S1P receptors in oligodendroglial development are called to shed the newly developed selective S1P receptor modulators, whose function is mostly examined regarding their immunomodulatory effects (Roggeri et al., 2020; Subei & Cohen, 2015).

The G protein–coupled receptor Gpr17 exerts an interesting regulatory role in OPC differentiation. At early stages of the lineage, this receptor acts as positive regulator of the process, while later on, its downregulation is required for further maturation, reviewed in (Wheeler & Fuss, 2016).

Extrinsic signals also activate the Notch and the Wnt pathways in oligodendrocytes and their role in OPC differentiation will be discussed below.

2.4.4. Opioids and nuclear receptors

Mu opioid receptors (MORs) stimulate DNA synthesis in OPCs and are downregulated throughout differentiation, thus suggesting a regulatory role in the process (Knapp et al., 1998; Tryoen-Tóth et al., 2000). Methadone, a MOR agonist, leads to increased OPC proliferation and maturation rates *in vitro* and increased myelin proteins in the developing rat brain when applied in therapeutic doses (Vestal-Laborde et al., 2014). In addition, it was found that MORs stimulate oligodendrogenesis from neural progenitor cell-derived NG2+ OPCs via the ERK and p38 signaling pathways (Hahn et al., 2010). While the role of MORs in oligodendrocyte development is not clear yet, the importance of Kappa opioid receptors (KORs) in the regulation of OPC differentiation and (re)myelination has been elucidated more

extensively (Wang et al., 2019). It was recently demonstrated that selective KOR agonism effectively promotes OPC proliferation, differentiation and (re)myelination (Du et al., 2016; Mei et al., 2016b). It has been suggested that KORs exert these effects either via sequential activation of the ERK signaling, CREB phosphorylation and myelin gene expression or via activation of the JAK2/STAT3 signaling (Borniger & Hesp, 2016).

Steroid hormone receptors are divided in the brain into mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), and they are among the nuclear receptor types which are implicated in oligodendroglial cell development (Vielkind et al., 1990). GR signaling modulates the expression of genes that regulate myelin gene expression (Barres et al., 1994; Kumar et al., 1989). In accordance, the glucocorticoid clobetasol was shown to promote OPC differentiation and remyelination in the EAE model via GR activation (Najm et al., 2015). Dexamethasone, another GR agonist also regulates OPC differentiation and myelination in a developmental stage-specific way (Almazan et al., 1986; Tsuneishi et al., 1991). Sex hormones especially progesterone, are also implicated in the regulation of oligodendroglial cell development. Specifically, it was demonstrated that both progesterone and nestorone promote OPC differentiation and remyelination in cuprizone-induced chronic demyelination (El-Etr et al., 2015). Similar effects mediated by testosterone and its synthetic analogue 7α-methyl-19-nortestosterone were attributed to activation of the androgen receptor (Bielecki et al., 2016; Hussain et al., 2013). Selective estrogen receptor modulators have been suggested as targets for the generation of myelin repair therapies, based on their capacity to promote OPC differentiation and myelination (Karim et al., 2019; Khalaj et al., 2016), however their effects at least in part are mediated via mechanisms other than estrogen receptor activation (Rankin et al., 2019).

The liver X receptor (LXR), which is activated by oxysterols, products of cholesterol oxidation, was similarly shown to promote OPC differentiation and remyelination on organotypic cerebellar slice cultures (Freemantle et al., 2013; Meffre et al., 2015). Functional analyses in cultured OPCs have revealed the positive regulatory role of the Retinoic x

Receptor gamma (RxRγ) in the process of differentiation (J. K. Huang et al., 2011). A few years later a pharmacological compound screening has revealed the potential of glucocorticoids to promote OPC differentiation with the best candidates promoting MBP expression via RxRγ activation (Porcu et al., 2015). The same year de la Fuente et al. provided evidence indicating RxRγ binding to several nuclear receptors, including the vitamin D receptor, which activates another positive regulatory signaling for OPC differentiation (de la Fuente et al., 2015).

Peroxisome proliferator-activated receptor gamma (PPARγ) activation promotes OPC differentiation and myelination via mitochondria-related mechanisms and regulation of Ca^{++} availability (Bernardo et al., 2009; Bernardo et al., 2013). Finally, thyroid hormone receptor expression increases with OPC maturation and its activation induces myelin gene expression and promotes differentiation and myelination, reviewed in (Marinelli et al., 2016).

2.4.5. Major regulatory pathways and metabolic control

The role of the Wnt pathway from OPC specification until myelination and remyelination has been extensively studied, reviewed in (Gaesser & Fyffe-Maricich, 2016; Guo et al., 2015; Xie et al., 2014). A few studies have shown that activation of the canonical Wnt/β-catenin pathway by extracellular Wnt ligands impairs OPC differentiation both in embryonic and early postnatal life (Fancy et al., 2009; Feigenson et al., 2009; Shimizu et al., 2005; Ye et al., 2009). In early postnatal life, this effect is at least in part mediated via recruitment of the TCF7L2 (also known as TCF4) as will be discussed later (Fancy et al., 2009). Several pharmacological and genetic studies revealed association between upregulation of the Wnt signaling and impaired myelin marker expression, as well as impaired differentiation (Azim & Butt, 2011; Fancy et al., 2011; Lang et al., 2013). The inhibitory role of the Wnt signaling in OPC differentiation is not only restricted in development but also regulates remyelination after white matter injury in the adult CNS (Fancy et al., 2011; Lee et al., 2015a; Lee et al., 2015b).

The Notch signaling is also a negative regulatory signaling for OPC differentiation which is activated by extracellular signals like Wnt signaling. Specifically, extracellular ligands induce proteolytic cleavage of the membrane-associated receptor Notch and its translocation to the nucleus (Louvi & Artavanis-Tsakonas, 2006). Treatment of OPC cultures with either Notch or its ligand Delta inhibited differentiation (Wang et al., 1998). Similarly, expression of the Jagged1 ligand by astrocytes in MS lesions led to inhibition of OPC maturation (John et al., 2002). Notch mediates its negative regulatory effects mainly via activation of the inhibitory factors Hes1 and Hes5 (Jarriault et al., 1998; Wang et al., 1998). Contactin is another Notch ligand, which is secreted by neuronal axons and activates a non-canonical Notch pathway with positive regulatory role in oligodendroglial cell differentiation (Hu et al., 2003). Notch signaling is crucial during development; however, its role in adult OPC differentiation is not yet clear. From one side, there are studies suggesting the existence of regulatory role of Notch1 signaling in oligodendrocyte development during CNS remyelination, while others failed to detect such a role when using animal models of demyelination (Stidworthy et al., 2004; Zhang et al., 2009). Possible suggested explanations for these contradictory results are either the investigation of different subpopulations from different CNS areas or the implication of different inflammatory signals from the environment (Kremer et al., 2011).

The Akt/mTOR pathway is a major regulatory pathway during oligodendroglial cell lineage progression both in development and adulthood, reviewed in (Gaesser & Fyffe-Maricich, 2016). Conditional knockout of the PI3K/AKT upstream inhibitor, phosphatase PTEN in mice leads to hypermyelination, without affecting OPC differentiation (Goebbels et al., 2010; Harrington et al., 2010). However, mTOR, a downstream effector molecule, positively affects the process as revealed by studies involving pharmacological inhibition and conditional knockout (Guardiola-Diaz et al., 2012; Tyler et al., 2009; Tyler et al., 2011; Wahl et al., 2014). mTOR1 and mTOR2 are two distinct protein complexes with distinct roles in oligodendroglial cell development. In the spinal cord, conditional knockout of both the mTORC1 protein subunit Raptor and the mTORC2 protein subunit Rictor, resulted in decreased OPC differentiation with the mTORC1 signaling ablation exerting a more potent effect (Bercury et

al., 2014). siRNA mediated knockdown of the same subunits induced reduction in myelin protein expression *in vitro* with mTORC2 alone controlling myelin gene expression at the mRNA level, and mTORC1 inducing MBP expression via an alternative mechanism (Tyler et al., 2009). Proteomic analysis has shown that during differentiation mTOR is required for the induction of proteins involved in cholesterol and fatty acid synthesis, the expression of cytoskeletal proteins, cell signaling components, and transcriptional factors (Tyler et al., 2011). However, a number of studies have shown that mTOR activity regulates OPC differentiation in different ways according to the developmental state of the cell and the location in the CNS, hence indicating implication of more than one regulatory mechanisms which have to be spatially and timely orchestrated in a delicate manner to ensure appropriate OPC differentiation and myelination, reviewed in (Gaesser & Fyffe-Maricich, 2016).

The Raf-MAPK-ERK1/2 signaling pathway has been described to positive regulate OPC differentiation. Several *in vitro* pharmacological studies showed impaired differentiation upon inhibition of MAPK-ERK activation, reviewed in (Gaesser & Fyffe-Maricich, 2016). Interestingly, the cytokine IL-17A, which is found in active MS lesions, increased the activity of both ERK1 and ERK2 and OPC differentiation in organotypic cerebellar slice cultures (Rodgers et al., 2015). In accordance, *erk2* deletion led to decrease in MBP expression and OPC differentiation in P10 mice and so did *b-raf* (upstream activation of the MAPK-ERK1/2 pathway) conditional knockout in P18 mice (Fyffe-Maricich et al., 2011; Galabova-Kovacs et al., 2008). However, some other studies failed to demonstrate ERK1- and ERK2-mediated effects on OPC differentiation *in vivo* indicating contribution of compensatory mechanisms which are not activated *in vitro*, as hypothesized by Gaesser and Fyffe-Maricich (Gaesser & Fyffe-Maricich, 2016; Ishii et al., 2012; Xiao et al., 2012). ERK2 is known to also positive regulate remyelination upon LPC-induced demyelination via control of the MBP translation (Michel et al., 2015). In addition, robust activation of ERK1/2 by pharmacological means boosted remyelination mainly via promotion of OPC differentiation (Najm et al., 2015). On the other hand, Suo et al. recently showed that pharmacological inhibition of the MAPK-ERK1/2 pathway promotes oligodendrocyte generation and recovery following EAE and cuprizone

mediated demyelination (Suo et al., 2019). These two studies also presented controversial MAPK-ERK1/2-mediated effects, which may be also attributed in time-specific effects, as proposed by the authors of the latter.

Lipids regulate oligodendroglial cell development and myelination and their metabolism has been suggested as target for myelin repair therapies, reviewed in (Marangon et al., 2020; Montani, 2020). Lipid homeostasis is regulated by mTOR and its downstream targets, namely SREBP cleavage activating protein (SCAP), and sterol responsive element binding proteins (SREBPs), reviewed in (Saxton & Sabatini, 2017). Already from their progenitor stage, cells of the oligodendroglial cell lineage start expressing immunoreactivity to antibodies recognizing specific lipids, as for example the A2B5 antibody which is specific for the gangliosides GD3, GT3, and O-acetylated GT3, whose expression ceases as the cells differentiate into mature oligodendrocytes (Jackman et al., 2009). Cholesterol plays an important role in myelin generation and dysfunctions in its metabolism have been associated with hypomyelinating disorders (Chrast et al., 2011). Regarding OPCs differentiation, cholesterol homeostasis, uptake and intracellular transport are essential for oligodendrocyte generation (Lin et al., 2017; Yu & Lieberman, 2013). In addition, pharmacological inhibition of the CYP51, TM7SF2 and EBP, intermediate enzymes of the cholesterol biosynthesis pathway and the subsequent accumulation of the 8,9-unsaturated sterol substrates of these enzymes was shown to promote OPC differentiation (Hubler et al., 2018; Rankin et al., 2019). Oxysterols, a group of cholesterol derivatives, promote myelin gene expression both in development and in the diseased CNS, thus acting as positive regulators of oligodendroglial cell differentiation (Meffre et al., 2015). *In vitro* studies also proposed fatty acids as important players in the regulation of the process, as seen by the promoting effects of docosahexaenoic acid (DHA) and the supportive role of the ratio of 1:3 linoleic acid to alpha linoleic acid (Hejr et al., 2017; Trapp & Bernsohn, 1978). Further highlightening the regulatory role of lipids, ganglioside GD1a was shown to promote OPC proliferation and differentiation, while galactocerebroside and sulfatide, the major galactosphingolipids in oligodendrocyte plasma membranes and myelin act as inhibitors of differentiation (Bansal et

al., 1999; Qin et al., 2017). Finally, the positive (although debated) effects of the sphingosine 1-phosphate receptor (S1PR) modulator, Fingolimod (FTY720), on OPC differentiation and process extension also underlines the regulatory role of sphingolipids (Cui et al., 2014; Miron et al., 2010).

2.4.6. Transcriptional control

The transition of OPCs to mature myelinating oligodendrocytes is controlled by several transcription factors, with some of them regulating also different stages of oligodendroglial cell development, reviewed in (Elbaz & Popko, 2019; Emery, 2010; Emery & Lu, 2015; Mitew et al., 2014; Tiane et al., 2019). OPCs are characterized by high levels of transcription factors such as Id2, Id4, Sox5, Sox6 and Hes5 which act as negative regulators of terminal differentiation. Id2 and Id4 mediate the anti-differentiation effects upon activation of BMPs (Cheng et al., 2007; Samanta & Kessler, 2004) and the G protein–coupled receptor Gpr17 (Chen et al., 2009). Physical interaction of Id4 and Id5 with the transcription factors OLIG1 and OLIG2 in the nucleus leads to inhibition of the activity of the latter two and differentiation blocking (Samanta & Kessler, 2004). Delta/Notch signaling activates Hes5 which in turn inhibits the transcription factor Sox10, thus preventing expression of myelin markers such as MBP and PLP (A. Liu et al., 2006; Samanta & Kessler, 2004; Wang et al., 1998). Sox5 and Sox6 bind directly to Sox10 targets, such as the MBP promoter, hence antagonizing its pro-differentiation effects (Stolt et al., 2006). Formation of a transcriptional complex between β-catenin and the transcription factor TCF7L2 mediates the inhibitory effects of the extracellular Wnt signaling, possibly via interactions with Id2 (Fancy et al., 2009; Fu et al., 2009; Ye et al., 2009). However, there are indications of a more complex role of β-catenin and TCF7L2 in the regulation of OPC differentiation, with the latter one being involved in chromatin remodeling (Emery & Lu, 2015; Ye et al., 2009).

OPC differentiation is also controlled by several transcription factors with positive regulatory properties. OLIG2 is a prominent positive regulator and although it is not clear yet how it exerts its functions, it has been shown that it recruits the chromatin-remodeling enzyme

brahma-related 1 (BRG1) to regulatory genes during differentiation including *Sox10*, *sip1/zhfx1b* and *myelin regulatory factor (myrf*) (Mei et al., 2013; Yu et al., 2013). OLIG1 deficiency has been associated with delay in OPC differentiation and delay or complete loss of mature markers such as MBP, however its expression merely regulates later stages of oligodendroglial cell development which correspond to myelin sheath formation (de Faria et al., 2014; Li et al., 2007; Niu et al., 2012; Xin et al., 2005). Myrf controls both the transition to mature oligodendrocytes and the process of myelination (Emery et al., 2009; Xin et al., 2005). According to Genome-wide binding studies (ChIP-Seq) Myrf directly targets genes regulating cytoskeletal dynamics, lipid metabolism, myelin protein genes such as *mbp* and *plp* and other transcriptional factors such as *nkx6.2* and *smad7* (Bujalka et al., 2013). Sox10 is constantly expressed throughout the whole oligodendroglial cell development and positively regulates terminal cell differentiation and myelination via binding to promoter regions of myelin genes (Hornig et al., 2013; Turnescu et al., 2018). Of note, synergistic effects between Myrf and Sox10 and a functional relationship with OLIG2 have been elucidated (Bujalka et al., 2013; Hornig et al., 2013; Yu et al., 2013). Zhfx1b positively regulates differentiation via antagonism of the inhibitory effects of Id2, Id4, and Hes5 and by extension BMPs and the Notch and Wnt signaling pathways (Weng et al., 2012). Smad7 is also induced by Zhfx1b and acts as a negative-feedback regulator of the BMPs (Weng et al., 2012). Similarly, Sox17 promotes cell cycle exit and differentiation via inhibition of the Wnt signaling (Chew et al., 2011; Sohn et al., 2006). Yin Yang 1 (YY1) transcription factor induces differentiation via inhibition of Id4 and the TCF7L2 downstream effector of the Wnt signaling (He, Sandoval, et al., 2007; Korinek et al., 1998). Finally, the achaete-scute homolog 1 (ASCL1/MASH1) promotes both oligodendroglial lineage progression and (re)myelination both in development and adulthood (Nakatani et al., 2013; Sugimori et al., 2008). *Ascl1* knockout leads to reduction in *nkx2.2* expression, whose absence is also related with blocking of OPC differentiation (Qi et al., 2001; Sugimori et al., 2008).

2.4.7. Epigenetic control

Epigenetic changes in the DNA are associated with oligodendroglial cell development, reviewed in (Emery & Lu, 2015; Fitzpatrick et al., 2015; Galloway & Moore, 2016; Tiane et al., 2019). DNA methylation, in particular CG methylation, regulates oligodendroglial cell development and myelination. This is reflected, among others, by alterations in the levels of DNA methylation in MS-affected brains, which are associated with hypomyelination (Huynh et al., 2014; Mastronardi et al., 2007). DNA methyltransferases (DNMTs) add a methyl-group to cytosine (5mC) which mostly leads to suppression of gene expression due to inability of transcription factors to bind to regulatory elements (Chen & Riggs, 2011). Neonatal inhibition of DNA methylation via a DNMT-inhibitor led to disrupted gliogenesis, reduced number of oligodendrocytes and caused hypomyelination in the rat optic nerve (Ransom et al., 1985). Similarly, genetic ablation of the *dnmt1* gene in embryonic OPCs led to hypomyelination which was associated with alternative splicing in genes related to lipid metabolism, myelination and cell cycle (Moyon et al., 2016). A less prominent role of DNA methylation in the adult remyelinating brain was shown upon LPS-mediated demyelination, where methylation was increased in differentiating oligodendrocytes (Moyon et al., 2017). OPC differentiation is highly influenced by ten-eleven translocation (TET) enzymes, which remove DNA methylation (Zhao et al., 2014). Knockdown of *tet* mRNA levels is associated with increased expression of inhibitory transcription factors such as Id2 and Hes5 (Zhao et al., 2014). Two histone-modification enzymes PRMT5 and SIRT2 promote initiation of OPC differentiation via induction of DNA methylation and gene silencing of the *Id2* and *Id4* and *pdgfrα* genes, respectively (Fang et al., 2019; J. Huang et al., 2011).

Histone modifications like (de)acetylation, methylation, ubiquitination, and phosphorylation regulate chromatin dynamics and structure, thus altering DNA accessibility for transcription factors and polymerases (Bannister & Kouzarides, 2011). Histone acetyltransferases (HATs) are the enzymes which establish acetylation, while histone deacetylases (HDACs) remove acetyl groups contributing to chromatin compaction, a conformation that prevents transcription (Bannister & Kouzarides, 2011; He et al., 2018). HDACs are predominantly expressed in early stages of oligodendroglial cell development, and their inhibition is

associated with decreased levels of OPC maturation and differentiation (Conway et al., 2012; Egawa et al., 2019; Marin-Husstege et al., 2002; Shen et al., 2005; Swiss et al., 2011). HDACs promote the switch from OPCs to mature oligodendrocytes via inhibition of expression of genes such as *Id2*, *Sox2* and *tcf7l2* that keep OPCs in a proliferative and undifferentiated state (Conway et al., 2012; Swiss et al., 2011). The pro-differentiation effects of these enzymes can also result via direct interaction with transcription factors and the formation of regulatory complexes. As example, YY1 is recruited via HDAC1 to the promoter of *Id2*, *Id4* and *hes5* and blocks their expression (He, Dupree, et al., 2007). HDAC mediated deacetylation of OLIG1 prevents interaction with the inhibitory Id2 transcription factor and as a result OLIG1 translocates to the nucleus to mediate OPC differentiation (Dai et al., 2015). In addition, the Wnt signaling is inhibited via interactions between the HDAC1/2 and TCF7L2 which lead to antagonism of the TCF7L2's binding site to β-catenin (Ye et al., 2009).

Histone methylation is another histone modification that has been associated with regulation of oligodendroglial cell differentiation. During the transition from OPC to mature myelinating oligodendrocytes, the activity of Histone H3 Lysine 9 (H3K9) methylation enzyme is higher (Liu et al., 2015). Accordingly, decreased histone methylation via genetic ablation or pharmacological inhibition of the protein arginine methyltransferases 1 and 5 (PRMT1 and PRMT5) lead to decreased OPC differentiation and hypomyelination (Hashimoto et al., 2016; Scaglione et al., 2018).

Moreover, ATP-dependent chromatin remodeling complexes which also alter histone accessibility and gene transcription have a regulatory role in oligodendroglial lineage progression. Such a complex, called brahma-related 1 (BRG1) regulates the expression of the *olig2* gene early in development via interaction with its promoter and as a positive feedback loop, is being in turn recruited by OLIG2 to enhance myelin related gene expression (Matsumoto et al., 2016; Yu et al., 2013). CDH7 is another ATP-dependent chromatin remodeler, and it is activated via OLIG2 and BRG1, predominantly in

differentiating oligodendrocytes, to promote myelin associated gene expression in synergy with Sox10 and to induce expression of pro-differentiation transcription factors, such as OLIG1 and Myrf (He et al., 2016).

Phosphorylation and dephosphorylation are key regulatory mechanisms throughout cell development which can modulate for example main regulatory pathways, such as the Raf-MAPK-ERK1/2 pathway (Fyffe-Maricich et al., 2011). As another example, the phosphorylation state of transcription factors, such as OLIG1 and ZFP24 determines the differentiation potential of OPCs (Elbaz et al., 2018; Niu et al., 2012).

Several miRNAs, small noncoding RNAs (ncRNAs) with negative regulatory roles in mRNA translation, represent another important regulatory player in OPC differentiation, reviewed in (Fitzpatrick et al., 2015; Galloway & Moore, 2016; Tiane et al., 2019). Interestingly, some miRNAs were shown to be differentially expressed in MS-affected brains from different stages of the disease progression (Junker et al., 2009; Noorbakhsh et al., 2011). An interesting example constitutes the miRNA-219 which along with the miRNA-338 is essential for cell cycle exit and transition from OPCs to mature oligodendrocytes (Dugas et al., 2010; Wang et al., 2017). miR-219, together with others was shown to be upregulated during OPC differentiation and also to cooperate with the miRNA-138, while boosting the expression of both was shown to be sufficient for the promotion of the process *in vitro* (Dugas et al., 2010; Ebrahimi-Barough et al., 2013; Shin et al., 2009; Wang et al., 2017). The absence of miRNA-219 in the CSF of MS patients has been proposed as diagnostic marker for the disease (Bruinsma et al., 2017). Of note, as some miRNAs promote cell differentiation, via for example suppression of negative regulatory transcription factors, such as Hes5 and Sox6 (Liu et al., 2017), some others like miR-205, miR-214 and miR-715 suppress the expression of myelin markers, such as MBP, O4 and myelin-associated oligodendrocyte basic protein (MOBP), thus inhibiting OPC maturation (Bauer et al., 2012; Bronstein et al., 2000; Letzen et al., 2010). In addition, two members of another subgroup of ncRNAs, called long noncoding RNAs (lncRNAs) have been associated with control of OPC differentiation. Specifically, lncOL1 promotes differentiation via inhibition of the expression of negative regulators and

lnc158 by regulating the transcription of the nuclear factor-IB (NFIB) (He et al., 2017; Li et al., 2018).

2.4.8. p57kip2: a potent inhibitor of OPC differentiation

The p57kip2 protein is a member of the Cip/Kip family of the cyclin-dependent kinase inhibitors (CKDI) originally described to negatively regulate the G1 phase of cell cycle progression in mammals (Besson et al., 2008; Lee et al., 1995; Matsuoka et al., 1995). Complexes of cyclins and cyclin-dependent kinases (CDK) are positive regulators of cell cycle progression, whereas binding of CDKIs blocks these complexes, thus exerting negative regulation. Cip/Kip protein family consists of three members, termed p21cip1, p27kip1 and p57kip2, which have been shown to bind to cyclins and CDK subunits and modulate their functions (Besson et al., 2008; Sherr & Roberts, 1995; Sherr & Roberts, 1999). p57kip2 binds to of G1- and S-phase CDKs, cyclin E-cdk2, cyclin D2-cdk4, and cyclin A-cdk2 and with lower affinity to the mitotic cyclin B-Cdc2 (Lee et al., 1995).

The p57kip2 gene (*cdkn1c*) is located in the chromosomal region 11p15.5 and the primary localization of the protein is in the nucleus. p57kip2 expression has a tissue-specific pattern during embryogenesis and adulthood, is required for embryonic development and its transcription is tightly regulated by factors important for this developmental stage such as Notch, MyoD, BMPs, and p73, reviewed in (Besson et al., 2008). The p57kip2 protein has a molecular weight of 57 kDa and consists of four different domains (Lee et al., 1995; Tokino et al., 1996). The CDK inhibitory domain containing cyclin and CDK binding sites is located in the N-terminus. The C-terminus consists of a glutamine- and threonine-rich QT domain which contains a nuclear localization sequence (NLS). The area between the N- and C- terminals is composed of a central proline-rich domain and an acidic domain containing glutamic or aspartic acidics in tandem repeats (Lee et al., 1995). N- and C- termini are homologous in mouse, rat and human p57kip2. The human p57kip2 protein differentiates by the lack of the proline-rich and acidic domains, which are substituted by a domain rich in proline-alanine repeats, called PAPA repeats domain (Tokino et al., 1996).

p57kip2 has an additional role as regulator of cytoskeletal dynamics via modulation of the RhoA/Rock/LIMK-1/cofilin pathway (Yokoo et al., 2003). Activation of the small GTPase RhoA, triggers the Rho-associated kinase (ROCK), which phosphorylates the downstream effector molecule LIM kinase-1 (LIMK-1). Upon phosphorylation, LIMK-1 is activated and via phosphorylation inactivates cofilin, a protein responsible for the depolymerization of actin, thus leading to assembly and stability of actin filaments (Arber et al., 1998). p57kip2 binds to LIMK-1, via its central proline-rich region and causes its translocation into the nucleus, promoting this way an increase in the levels of actin disassembly (Yokoo et al., 2003).

A novel role of p57kip2 as intrinsic negative regulator of peripheral glial cell differentiation has been suggested upon observation of its transient down-regulation following sciatic nerve crush (Küry et al., 2002). In this respect, it was shown that p57kip2 negatively affects myelinating glial cell differentiation both in PNS and CNS and that it controls glial fate decision by adult neural stem cells (Heinen et al., 2008; Jadasz et al., 2012; Kremer et al., 2009). Furthermore, transient p57kip2 down-regulation in a MOG-induced EAE was accompanied by relocation to the cytoplasm (Kremer et al., 2009). The same study also reported increased maturation and differentiation in OPC cultures upon transient p57kip2 down-regulation, thus strengthening the notion of its negative regulatory role in oligodendroglial differentiation and maturation. In this context, the negative impact of p57kip2 on differentiation was shown to be associated with alterations in p57kip2 subcellular localization during rat and human oligodendroglial differentiation *in vitro* (Göttle et al., 2015). Enforced nuclear export of p57kip2 was correlated with enhanced myelin expression, cell maturation and myelination *in vitro*. The same study suggested that p57kip2's inhibitory role is dependent on binding partners such as LIMK-1, CDK2, Mash1 and Hes5, which leads to control of their subcellular distribution and/or activity. Of note, p57kip2's nuclear exclusion does not seem to exert the same regulatory effects on cultured hippocampal adult neural stem cells (aNSCs) (Jadasz et al., 2018). Finally, a recent *in vivo* study demonstrated that in the healthy adult CNS, *p57kip2* knockdown promotes adult neural stem cell survival and differentiation towards myelinating oligodendrocytes at the expense of the astrocytic lineage,

while upon injury these effects were less prominent and the cells showed higher vulnerability (Beyer et al., 2020). Taken together, these studies suggest a therapeutic potential of the modulation of p57kip2's protein subcellular localization and activity for functional restoration of myelin sheaths in demyelinating diseases.

2.5. Development of therapeutic approaches for myelin repair

2.5.1. Oligodendrocyte and myelin plasticity

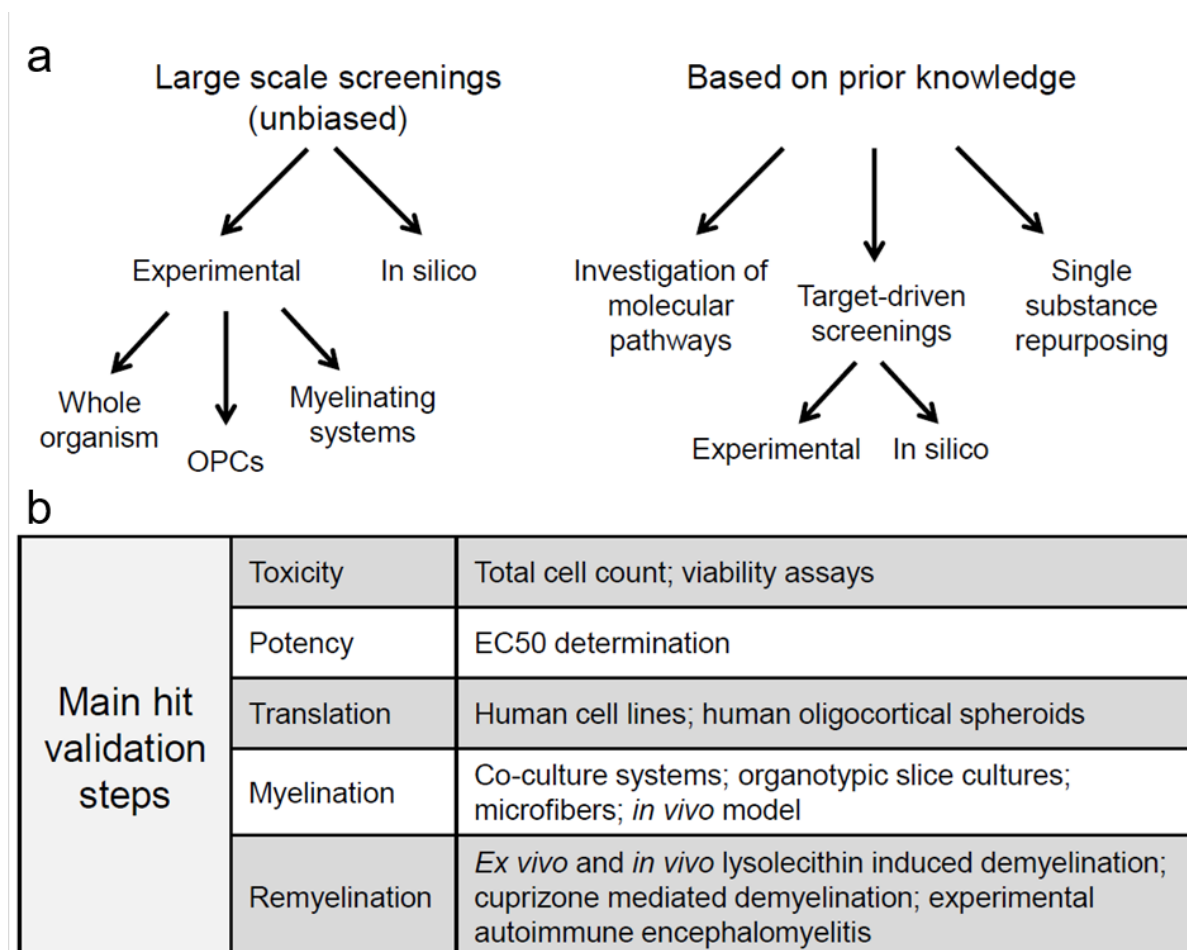
In the previous chapters the factors that regulate oligodendroglial cell lineage progression and myelin formation both during development and adulthood have been discussed. Fine orchestration of them contributes to preservation of oligodendrocyte, myelin, and neuronal network homeostasis in the CNS in response to development, learning and injury. There is evidence for ongoing oligodendrogenesis throughout lifetime, mainly upon differentiation of adult OPCs. Under physiological conditions up-regulation of this process is correlated with myelin remodeling in an experience-dependent manner, as for example during motor skill learning (Dawson et al., 2003; Hill et al., 2018; Hughes et al., 2018; McKenzie et al., 2014; Xiao et al., 2016). An example of how oligodendrocyte lineage plasticity ensures proper CNS development constitutes the study of Kessaris et al., where upon ablation of mouse telencephalic OPCs, recovery and normal development took place following migration of OPCs from other areas (Kessaris et al., 2006). Similarly, in the adult brain, OPCs create their own territory of surveillance via self-repulsion and in case of OPC loss due to cell death or differentiation they migrate and proliferate to repopulate the vacant areas, thus participating to myelin repair upon CNS injury (Hughes et al., 2013). In addition, it has been suggested that adult OPCs, although pretty much committed to the oligodendroglial fate, they have the ability to adopt a different cell fate in response to injury (Cassiani-Ingoni et al., 2006; Nishiyama et al., 2009; Zawadzka et al., 2010), and even be reprogrammed to multipotent neural stem-like cells upon pharmacological inhibition of HDAC activity (Lyssiotis et al., 2007). Taken together, the last studies suggest that OPC programming and transplantation can constitute a therapeutic strategy for CNS injury, however this approach still faces crucial limitations (Lopez Juarez et al., 2016). In addition, as discussed earlier, the rate-limiting

factor for efficient myelin repair is not the availability of OPCs, but rather their ability to differentiate and give rise to functional myelinating oligodendrocytes, which deteriorates with age and under pathophysiological conditions due to lack of stimulatory cues or presence of inhibitory signals. Therefore, the development of therapeutic interventions which aim to boost the differentiation of OPCs seems to be one of the most promising approaches for myelin repair. In this respect, it is important to keep in mind that although myelination and remyelination share many common aspects, the latter cannot be considered as recapitulation of the first one since there are differences in their outcome, such as the thickness of the myelin sheaths, and also in their regulation (Franklin & ffrench-Constant, 2008; Taveggia et al., 2010). Hence, understanding the signals that regulate oligodendroglial cell development and plasticity both in development and in adulthood is important for deciphering myelin repair processes and the development of effective therapies.

2.5.2. Phenotypical screenings for pharmacological modulation of OPC differentiation

Although there is ongoing progress in the development of active compounds with neuroregenerative properties, with some of them having already entered clinical trials, induction of sufficient remyelination as therapeutic approach has not been yet achieved (Kremer et al., 2019b; Kremer et al., 2019c; Küry et al., 2018). Many research groups now are working towards the same direction using related approaches. On one hand, there are the unbiased large scale screening approaches, experimental or in silico, which use large numbers of available active compounds and the existing knowledge in different contexts about them, to identify and repurpose a few of them for myelin repair. On the other hand, we have approaches which require prior knowledge about regulatory molecular mechanisms. These approaches can be assigned in three different categories, (i) target-driven screenings, which aim to identify compounds that modulate molecular pathways with known implication in oligodendroglial development, (ii) pharmacological approaches, which aim to determine whether or how specific molecular pathways are implicated in the process and whether their

modulation can constitute a therapeutic approach, and (iii) single substance repurposing approaches (Fig. 4).



Main hit validation steps		
	Toxicity	Total cell count; viability assays
	Potency	EC50 determination
	Translation	Human cell lines; human oligocortical spheroids
	Myelination	Co-culture systems; organotypic slice cultures; microfibers; *in vivo* model
	Remyelination	*Ex vivo* and *in vivo* lysolecithin induced demyelination; cuprizone mediated demyelination; experimental autoimmune encephalomyelitis

Figure 4. Overview of the existing pharmacological approaches for the identification of pro-myelinating compounds and the study of molecular mechanisms underlying myelin repair (a). Brief presentation of the main hit validation steps, following a screening for pro-myelinating compounds (b).

Most of the phenotypic compound screenings aim to identify substances able to boost oligodendroglial cell differentiation, a pivotal process for myelin repair. Several cell culture based studies have been designed to screen pharmacological compounds on primary OPC cultures (Chen et al., 2017; Deshmukh et al., 2013; Eleuteri et al., 2017; Lariosa-Willingham et al., 2016a; Rosler et al., 2016), cell lines (Joubert et al., 2010; Peppard et al., 2015; Porcu et al., 2015), neural precursor cells (NPCs) (Cui et al., 2019; Guo et al., 2018; Suo et al., 2019) and pluripotent mouse epiblast stem cell (EpiSC)-derived OPCs (Allimuthu et al., 2019; Elitt et al., 2018; Hubler et al., 2018; Najm et al., 2015). Most of these screenings used

MBP expression as readout to identify compounds that promote OPC differentiation (Allimuthu et al., 2019; Chen et al., 2017; Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Hubler et al., 2018; Lariosa-Willingham et al., 2016a; Mei et al., 2014; Najm et al., 2015; Porcu et al., 2015; Suo et al., 2019). Alternatively, morphological parameters (Joubert et al., 2010; Peppard et al., 2015) and metabolic activity were used by others for the same purpose (Eleuteri et al., 2017). Of note, a number of cell-based screenings have been designed for direct identification of compounds that promote myelination (Lariosa-Willingham et al., 2016a; Mei et al., 2014) and some others for compounds that increase OPC viability as alternative means of boosting remyelination (Elitt et al., 2018; Rosler et al., 2016).

Of note, the use of zebrafish, as whole organism, has been demonstrated to be effective for the study of oligodendroglial cell differentiation and myelination *in vivo*, reviewed in (Czopka, 2016). In this respect, pharmacological screening platforms have been developed for compounds that promote oligodendroglial cell development and myelin production both in development (Buckley et al., 2010; Early et al., 2018; Preston et al., 2019) and as rescuing effect in disease (Diamantopoulou et al., 2019).

In silico drug repurposing approaches apply analytical methods to data from available databases to elucidate associations between pathophysiological conditions and drugs. Molecular in silico approaches are based on drug- and disease-focused databases and tools that link drugs and disease to identify drug-induced changes in transcriptomic and genomic signatures in the concept of a disease. In silico virtual screenings are complementary to experimental high-throughput screening (HTS) since they are often producing false positive hits but their contribution is valuable since they are cheap and efficient (Cha et al., 2018; Wang et al., 2019). Recently, a virtual target-driven HTS of approximately 130.000 lead like compounds for the identification of agonists with binding affinity to the GPR17 protein, an inhibitor of OPC differentiation and myelination led to identification of 29 compounds belonging to 3 distinct families. After focusing on one of them, the library was expanded via cheminformatical methods to increase diversity and drug-likeness. Ultimately, some of the

top hits turned out to be commercially available, the pro-oligodendroglial properties of some of them were shown experimentally and one of them delayed the onset of EAE *in vivo* (Parravicini et al., 2020). Another recent study that highlights the importance of in silico screening involves the use of public microarray results from the GEO database and interrogation of the connectivity map (CMAP) (Zhu et al., 2019). This screening led to the identification of differential gene expression in corpus callosum from naïve and cuprizone-treated mice and gave rise to a list of compounds with pro-remyelinating properties, the best of which were confirmed and validated experimentally *in vitro* and *in vivo* (Zhu et al., 2019).

The choice of the compound library which will be tested in a phenotypic screening is a decisive factor for the efficiency of the approach. The decision is being made after taking into account criteria like molecular and pharmacological properties of the compounds, the size and the cost of the library and its previous applications. A few reviews can provide guidance and easy to use tools, like cheminformatics software tools, for careful selection of a compound library (Dandapani et al., 2012; Volochnyuk et al., 2019). As example, the Bemis-Murcko scaffold analysis is a tool that determines the structural and topological uniqueness of each compound within a collection or upon comparison of more than one collections providing knowledge about the actual number of unique molecules the purchaser will receive (Bemis & Murcko, 1996). ZINC constitutes a useful free database of purchasable compounds for virtual screening and also a catalog of vendors for commercially available compound libraries (Sterling & Irwin, 2015).

Large scale pharmacological screenings are succeeded by a row of validation steps which lead to confirmation of hits, as well as determination of their pharmacological properties, toxicity, translational potential to the human system and effects on myelination and remyelination (Fig. 4b). Toxic effects in cell culture can be detected by a diminish in the total cell count (Allimuthu et al., 2019; Elitt et al., 2018; Najm et al., 2015) or via use of viability assays based on luminescent indicators of metabolic activity (Peppard et al., 2015; Rosler et al., 2016). Studies commonly include validation and ranking of primary hits according to their

potency by means of half maximal effective concentration (EC50) determination (Chen et al., 2017; Deshmukh et al., 2013; Lariosa-Willingham et al., 2016a, 2016b; Najm et al., 2015; Parravicini et al., 2020; Peppard et al., 2015; Porcu et al., 2015; Rosler et al., 2016). To assess the translational potential of the primary hits, cells of human origin, as for example human OPCs generated from skin fibroblast-derived human induced pluripotent stem cell (iPSC) line and human embryonic stem cell (hESC) lines H7 and H9 (Najm et al., 2015), human glioma cell line GBM528 (Hubler et al., 2018), and human Pelizaeus-Merzbacher disease (PMD) patient iPSC-derived cells used for generation of oligocortical spheroids (Elitt et al., 2018) have been used to reproduce the effect on cells of rodent origin. Hit validation in terms of the impact on developmental myelination is a crucial step for the determination of the efficacy of a drug for use in myelin repair. For this purpose, neuron- oligodendrocyte co-culture systems (Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Parravicini et al., 2020; Suo et al., 2019), electrospun microfibers (Allimuthu et al., 2019; Elitt et al., 2018; Hubler et al., 2018), organotypic cerebellar slices (Eleuteri et al., 2017; Najm et al., 2015) and *in vivo* drug administration (Najm et al., 2015) have been used to detect beneficial effects on developmental myelination upon hit compound application. Finally, to demonstrate remyelination promoting properties of hit compounds LPC-mediated demyelination has been used both *ex vivo* (Eleuteri et al., 2017) and *in vivo* (Hubler et al., 2018; Mei et al., 2014; Najm et al., 2015). More often, the cuprizone model of demyelination (Chen et al., 2017; Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Suo et al., 2019; Zhu et al., 2019) or the EAE model for MS (Deshmukh et al., 2013; Najm et al., 2015; Parravicini et al., 2020; Suo et al., 2019) have been used for the same purpose. In some studies, even more than one models were combined to demonstrate detailed effects of a treatment (Deshmukh et al., 2013; Najm et al., 2015; Suo et al., 2019).

2.5.3. Target-driven screenings and singe substance based studies

Large scale screenings are mostly biased free and aim to identify novel compounds with desired properties and whose molecular targets can then be used to identify cellular pathways that are implicated in the process of interest. A different approach with a significant

contribution to the study of oligodendrocyte biology and myelin repair, as well as to the identification of modulatory compounds, is the development of targeted screenings or single substance testing that are based either on hypothesis or prior knowledge about the implication of specific molecular mechanisms in the aforementioned processes.

Targeted screens for pro-myelinating compounds are based on prior knowledge of the implication of specific signaling pathways or molecules in oligodendroglial cell development, as for example GPCRs or the RhoA//ROCK and PKCa signaling pathways and the use of known modulators of them for the detection of pro-oligodendroglial effects (Gonzalez et al., 2016; Mei et al., 2016a). Interestingly, information about hit compounds from a few biased-free high content screenings (Mei et al., 2014; Najm et al., 2015; Porcu et al., 2015) led to development of follow-up targeted screenings, further characterization of their mode of action and identification of additional compounds with pro-myelinating properties (Ashikawa et al., 2016; Rankin et al., 2019).

Many studies have used bioactive compounds to either determine whether specific molecular pathways can become targets of pro-myelinating drugs or to shed light on their implication in oligodendroglial biology or myelination. Among the molecules and signaling/processes that have been identified as druggable targets in this context are K_{ATP} channels and cell hyperpolarization (Fogal et al., 2010), major chromatin regulators BET and lysine acetylation (Gacias et al., 2014), ROCK and the Rho-A/ROCK signaling (Pedraza et al., 2014), cAMP-specific phosphodiesterase 7 (PDE7) and glycogen synthase kinase 3 (GSK3) (Medina-Rodríguez et al., 2013; Medina-Rodríguez et al., 2017), neurotrophin brain-derived neurotrophic factor (BDNF) (Fletcher et al., 2018), BMP4 (Govier-Cole et al., 2019), mTOR (Tyler et al., 2009), cannabinoid receptors (Gomez et al., 2011), thyroid hormone (Hartley et al., 2019) and GPR17 (Hennen et al., 2013; Merten et al., 2018).

Finally, another approach for the identification of pro-myelinating drugs is the investigation of the effects on oligodendroglial biology and myelination of agents with known immunomodulatory or neuroprotective properties, with some of them being approved or

suggested candidates for neurodegenerative diseases, such as MS. Interesting examples in this context are olexisome, a suggested candidate agent for the treatment of amyotropic lateral sclerosis with neuroprotective and neuroregenerative properties (Magalon et al., 2012), the approved immunomodulatory drugs for RRMS fingolimod and teriflunomide (Göttle et al., 2018; Miron et al., 2008; Miron et al., 2010; Zhang et al., 2015), aspirin which has neuroprotective effects and a broad spectrum of therapeutic applications (Chen et al., 2014), Vitamin D which has reported immunomodulatory and protective effects in EAE (de la Fuente et al., 2015; Shirazi et al., 2015) and the anti-psychotic drug quentiapine (Xiao et al., 2008; Zhang et al., 2012).

2.6. Aim of the study

Multiple sclerosis is among the most debilitating autoimmune demyelinating diseases, affects a great number of mostly young people, and leads to permanent neurological deficits (Noseworthy, 1999). The currently available therapies are focused on immunomodulation, but they fail to invert the already existed damage, which develops as neurodegeneration progresses due to the absence of myelin sheaths and oligodendrocyte loss (Kremer et al., 2019b). Adult OPCs remain an undifferentiated state throughout adulthood and in response to injury or disease they are getting activated to eventually differentiate into myelinating oligodendrocytes which give rise to new myelin sheaths, a process called remyelination (Dawson et al., 2003). The innate capacity of the CNS for myelin repair is, however, not sufficient and declines as the disease progression worsens (Franklin & ffrench-Constant, 2017). The rate-limiting event for remyelination is the inability of the OPCs to differentiate due to lack of stimulatory cues or the presence of inhibitory signals (Gruchot et al., 2019). Over the past few years many scientific approaches have led to the identification of pharmacological compounds which boost OPC differentiation and eventually myelin repair, with some of them already been in clinical trials (Deshmukh et al., 2013; Hubler et al., 2018; Najm et al., 2015). Despite all this progress, an effective treatment has not yet been developed.

This study is based on knowledge regarding the inhibitory role of the p57kip2 protein in oligodendrogenesis, which is only exerted when the protein is localized in the nucleus, while nuclear exclusion is associated with later stages of development and initiation of differentiation (Göttle et al., 2015). p57kip2's subcellular localization was used as readout for the development of a phenotypic screening for compounds with the potential to promote OPC differentiation and myelin repair and knowledge about the hit compounds was used to shed light on the underlying mode of actions.

3. MATERIALS AND METHODS

3.1. Material

3.1.1. Animals and human tissue

The animals used for this study were ordered from the Janvier Labs (Le Genest-Saint-Isle, France) and/or bred within the animal facility of the Heinrich-Heine-University of Düsseldorf, under pathogenic free standard conditions (temperature 21ºC, humidity 50 (+/-5)%, 12 hours (h) light/12 h dark cycle. During *in vivo* experiments, animals were kept in specified groups in standard cages with water and food supplies available ad libitum. For the generation of the primary oligodendroglial cell and organotypic cerebellar slice cultures Wistar rats of either sex on postnatal day 0 or 1 (P0 or P1) and P7, respectively, were used. For the cuprizone-mediated demyelination seven-week-old female C57BL/6 mice ordered from Janvier Labs, were directly split into groups, and left for one week to get acquainted to the environment before the initiation of the experiment.

Ethical approval for the generation of rodent primary oligodendroglial cell and organotypic cerebellar slice cultures was provided by the ZETT (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben; O69/11, V54/09). The cuprizone-mediated demyelination animal model used in this study was approved by the authorities at LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; Az.: 81-02.04.2019.A203) and carried out with ethical care.

The human OPCs derived from fetal tissue supplied by a National Institutes of Health (NIH) supported program, called the U of Washington. Second trimester (14-17 weeks) fetal samples were obtained from the University of Washington Birth Defects Research Laboratory (MP-37-2014-540; 13-244-PED; eReviews_3345).

3.1.2. Chemicals

3.1.2.1. Reagents/buffers/chemicals

Reagent/buffer/chemical	Supplier
4,6-diamidino-2-phenylindole (DAPI)	Roche
Agarose, UltraPure Low Melting Point	Thermo Fisher Scientific

Ringer solution	Fresenius Kabi
Magnesium chloride (MgCl2·6H2O)	SERVA
HEPES buffer 1 M	Thermo Fisher Scientific
Bovine Albumin Fraction V (BSA; 7.5% solution)	Thermo Fisher Scientific
Bovine Albumin Fraction V (BSA; powder)	Carl Roth
Citifluor AF1 Cat #17970-100	Electron Microscopy Sciences
Dimethylsulfoxide (DMSO)	Sigma-Aldrich
Isoflurane	Piramal
Ethanol 96%	Merck
Skimmed milk powder	Carl Roth
Trypan blue, 0.4%	Lonza
Paraformaldehyde (PFA)	Merck
Sodium chloride (NaCl)	Merch
Trishydroxymethylaminomethan (Tris)	Carl Roth
Triton X-100	Sigma-Aldrich
Tween 80	Sigma-Aldrich
Tween 20	Sigma-Aldrich
0.2% (w/w) cuprizone containing diet pellets Cat#TD.140803	Envigo
Cuprizone CAS 370-81-0	Sigma-Aldrich
V1534 implemented Cat#V1534	SSNIFF
Prestwick Chemical Library Cat#PCL1280.10-50-G96	Prestwick
Fingolimod (FTY720) CAS 402616-26-6	Echelon Biosciences
Parbendazole CAS 14255-87-9	MedChem Express
Danazol CAS 17230-88-5	Sigma-Aldrich
Poly-D-lysine coated (PDL)	Sigma-Aldrich
Trypsin-EDTA	Capricorn Scientific
RIPA buffer	Cell Signaling Technology
HALT™ Protease-/Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific
EDTA	Thermo Fisher Scientific
Bolt 12% Bis-Tris Plus mini protein gel	Thermo Fisher Scientific
Nitrocellulose membranes	Thermo Fisher Scientific
1x ReBlot Plus Strong Solution	Merck Millipore
10x Bolt Sample Reducing Agent	Thermo Fisher Scientific
4x Bolt LDS Sample Buffer	Thermo Fisher Scientific
20x Bolt MOPS SDS Run Buffer	Thermo Fisher Scientific
Pierce™ 1-Step Transfer Buffer	Thermo Fisher Scientific

Magic marker (MM)	Thermo Fisher Scientific
SeeBlue™ Pre-stained Protein Standard	Thermo Fisher Scientific
Pierce™ Lane Marker Non-Reducing Sample Buffer	Thermo Fisher Scientific
Fetal bovine serum (FBS)	Lonza, Basel; Capricorn Scientific
Superglue	UHU
Heat inactivated horse serum	Thermo Fisher Scientific
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich
Hank's Balanced Salt Solution (HBSS)	Thermo Fisher Scientific
Normal goat serum (NGS)	Sigma-Aldrich
Trypsin	Thermo Fisher Scientific
Poly-L-lysine (PLL)	Sigma-Aldrich
Poly-D-lysine (PDL)	Sigma-Aldrich
ECM-gel from Engelbreth-Holm-Swarm murine sarcoma	Sigma-Aldrich
Sterile saline solution (0,9% NaCl)	Fresenius Kabi
Sucrose	Carl Roth
Tissue-Tek OCT compound	Sakura Finetek
Acetone	Merck
Normal donkey serum	Sigma-Aldrich
Shandon™ Immu-Mount	Thermo Fisher Scientific
2-Methylbutane	Merck

3.1.2.2. Media for cell culture

Medium	Supplier/Composition
Dulbecco's modified eagle medium (DMEM)	Thermo Fisher Scientific
Dulbecco's modified eagle medium GlutaMax-I (DMEM GlutaMax-I)	Thermo Fisher Scientific
Minimum essential medium (MEM), c/HEPES, s/L-Glu	Thermo Fisher Scientific
Leibovitz's medium L-15	Thermo Fisher Scientific
Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham	Sigma-Aldrich
Rat cortical tissue digestion medium	MEM implemented with 40 µg/ml DNAse; 0.24 mg/ml L-cystein; 30 U/ml papain (all Sigma-Aldrich)
Trypsin inhibitor solution	Leibvitz's medium L-15 implemented with 1 mg/ml

	trypsin inhibitor (Sigma-Aldrich); 50 mg/ml BSA (Carl Roth); 40µg/ml DNase I type IV (Sigma-Aldrich)
Mixed glial culture medium	DMEM implemented with 10% FBS; 4 mM L-glutamine (Thermo Fisher Scientific); 100 U/ml penicillin/0.1 mg/ml streptomycin (Thermo Fisher Scientific)
Rat oligodendroglial cell culture medium (SATO)	DMEM GlutaMax-I implemented with 5 µg/ml bovine insulin; 50 µg/ml human transferrin; 100 µg/ml BSA (Carl Roth); 6.2 ng/ml progesterone; 16 µg/ml putrescine, 5 ng/ml sodium selenite; 400 ng/ml T3 (tri-iodo-thyronine); 400 ng/ml T4 (thyroxin; all Sigma-Aldrich); 4 mM L-glutamine; 100 U/ml penicillin/0.1 mg/l streptomycin (both Thermo Fisher Scientific)
Oligodendroglial cell culture differentiation medium	SATO implemented with 0.5% FBS
Slice culture medium	50% MEM; 25% HBSS +/+; 25% heat inactivated horse serum; implemented with 100 U/ml penicillin/0.1 mg/ml streptomycin (Thermo Fisher Scientific); 5 mg/ml D-glucose monohydrate (Merck Millipore)
Human telencephalic tissue digestion medium	0.25% Trypsin (Thermo Fisher Scientific); 25 µg/mL DNase I (Roche, Laval, Canada)
Human oligodendroglial cell culture medium	DMEM-F12 media supplemented with N1 (Sigma-

	Aldrich); B27 supplement (Sigma-Aldrich); 10ng/ml PDGF-AA; 10 ng/ml bFGF (Sigma-Aldrich)

3.1.2.3. Commercial Assays

Assay	Supplier
DC™ Protein Assay Kit II Cat#5000112	BIO-RAD
Pierce™ Reversible Protein Stain Kit Cat#24580	Thermo Fisher Scientific
SuperSignal™ West Pico PLUS Chemiluminescent Substrate Cat#34579	Thermo Fisher Scientific

3.1.2.4. Antibodies

Antibody	Supplier	Identifier
Rabbit polyclonal anti-p57kip2 (1:250)	Sigma-Aldrich	Cat#P0357, RRID:AB_260850
Rabbit polyclonal anti-p38 (1:1000)	Cell Signaling Technology	Cat#9212, RRID:AB_330713
Rabbit polyclonal anti-p-p38 (1:1000)	Cell Signaling Technology	Cat#9211, RRID:AB_331641
Rabbit polyclonal anti-NF (1:1000)	Abcam	Cat#ab8135, RRID:AB_306298
Rabbit polyclonal anti-GSTpi (1:2000)	Enzo Life Sciences	Cat#ADI-MSA-101-E, RRID:AB_2039147
Rat monoclonal anti-MBP (1:250)	Bio-Rad	Cat#MCA409S, RRID:AB_325004
Rat anti-PLP monoclonal (1:250)	Kind gift from B. Trapp and R. Dutta, Dept. of Neurosciences, Cleveland Clinic, OH	(Chen et al., 2015)
Mouse monoclonal anti-GAPDH (1:5000)	Merck Millipore	Cat#MAB374, RRID:AB_2107445
Mouse monoclonal anti-OLIG2 (1:500)	Merck Millipore	Cat#MABN50, RRID:AB_10807410
Mouse monoclonal anti-O4 (1:200)	R and D Systems	Cat#MAB1326, RRID:AB_357617

Mouse monoclonal anti-CC1 (1:1000 ICC, 1:500 IHC)	GeneTex	Cat#GTX16794, RRID:AB_422404
Mouse monoclonal anti-MOG (1:500)	Merck Millipore	Cat#MAB5680, RRID:AB_1587278
Goat polyclonal anti-PDGFR (1:250)	Neuromics	Cat#GT15150, RRID:AB_2737233
Hybridoma anti-GalC IgG3 (1:50)	Jack. P. Antel, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada	(Kremer et al., 2016b)
Goat anti-rabbit Alexa Fluor 594 (1:500)	Thermo Fisher Scientific	Cat#A-11037, RRID:AB_2534095
Goat anti-rabbit Alexa Fluor 405 (1:500)	Thermo Fisher Scientific	Cat#A-31556, RRID:AB_221605
Goat anti-rabbit Alexa Fluor 488 (1:200)	Thermo Fisher Scientific	Cat#A-11008, RRID:AB_143165
Goat anti-rat Alexa Fluor 488 (1:500 ICC, 1:200 IHC)	Thermo Fisher Scientific	Cat#A-11006, RRID:AB_2534074
Goat anti-mouse Alexa Fluor 647 (1:500)	Thermo Fisher Scientific	Cat#A32728, RRID:AB_2633277)
Goat anti-mouse Alexa Fluor 488 (1:500 ICC, 1:200 IHC)	Thermo Fisher Scientific	Cat#A32728, RRID:AB_2633277
Donkey anti-goat Alexa Fluor 488 (1:200)	Thermo Fisher Scientific	Cat#A-11055, RRID:AB_2534102
Goat anti-mouse IgM-Alexa Fluor 647 (1:500)	SouthernBiotech	Cat#1021-31, RRID:AB_2794254
Goat anti-mouse IgG3-Alexa Fluor 488 (1:500)	Thermo Fisher Scientific	Cat#A-21151, RRID:AB_2535784
Anti-rabbit IgG, HRP-linked (1:2000)	Cell Signaling Technology	Cat#7074, RRID:AB_2099233
Anti-Mouse IgG (H+L), made in horse (1:5000)	Vector Laboratories	Cat#PI-2000, RRID:AB_2336177
Hoechst 33258 (1:1000)	Thermo Fisher Scientific	Cat#H3569, RRID:AB_2651133

3.1.3. Culture flasks, plates, and dishes

Flask/plate/dish	Supplier
T-75 cell culture flask	Greiner Bio-One
Cell Culture Dish, ø 100 mm	Greiner Bio-One
Cell Culture Multiwell Plate, 24 Well	Greiner Bio-One
Cell Culture Multiwell Plate, 12 Well	Greiner Bio-One
Microplate, 96 well, PS, U-Bottom, Clear	Greiner Bio-One
96-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid	Corning
ø 13 mm microscope cover glasses	Hecht Assistent
Cell culture inserts, hydrophilic PTFE, pore size: 0.4 µm, diameter 30 mm	Merck Millipore

3.1.4. Material for *in vivo* experimentation and immunostaining

Material	Supplier
SuperFrost Plus microscope slides	Thermo Fisher Scientific
Microscope slides (76 x 26 mm)	Engelbrecht
Mini Pap Pen	Thermo Fisher Scientific
Butterfly needles	BD Vacutainer
1 ml syringe	Braun
Needles (0.45 x 10 mm)	BD Microlance 3

3.1.5. Hardware and software

3.1.5.1. Technical devices

Device	Supplier
Eppendorf Centrifuge 5804	Eppendorf
Thermo Scientific Fresco 21 Microcentrifuge	Thermo Fisher Scientific
Heraeus Megafuge 3.0R Refrigerated Centrifuge	Thermo Fisher Scientific
New Brunswick Excella® E24/E24R	Eppendorf
Heraeus Hera Safe incubator BBD 6220	Thermo Fisher Scientific
Infinite M200 Pro plate reader	TECAN
SonopulsHD2070 ultrasonic homogenizer	Bandelin
Zeiss Axionplan2 microscope	Carl Zeiss Microscopy
Safe 2020 Class II Biological Safety Cabinets	Thermo Fisher Scientific
CLSM 510 confocal microscope	Carl Zeiss Microscopy
XCell SureLock Mini-Cell Electrophoresis System	Thermo Fisher Scientific
Power Blotter–Semi-dry Transfer System	Thermo Fisher Scientific

Fusion FX	Vilber Lourmat
BD Pathway 855 High-Content Cell Analyzer	BD Biosciences
HM 650V microtome	Thermo Fisher Scientific
Leica CM3050S cryostat	Leica
Light microscope	Leica
Stereoscope	Olympus

3.1.5.2. Software/algorithm

Software/algorithm	Supplier	Identifier
ImageJ BioVoxxel software	BioVoxxel Toolbox	http://www.biovoxxel.de/ RRID:SCR_015825
Fusion FX software	Vilber Lourmat	https://www.vilber.com/fusion-fx/#specs/
Attovision v.1.7.1 software package	BD Biosciences Systems	https://www.bdbiosciences.com/en-us/site-terms-and-conditions/ RRID:SCR_014315
ZEN 2 Blue Edition software	Carl ZEISS Microscopy	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html RRID:SCR_013672
Matlab Software R2015a	Mathworks, Inc	http://www.mathworks.com/products/matlab/ RRID:SCR_001622
GraphPad Prism 8.0.2 software	GraphPad Prism	http://www.graphpad.com/ RRID:SCR_002798
Custom Code	(Manousi et al., 2021)	https://github.com/anastasiamanousi/p57kip2-translocation-Manousi-et-al.-2020
G*Power 3.1.9.2 software	Heinrich-Heine-University	(Faul et al., 2009)
Microsof Excel	Microsoft office	https://www.microsoft.com/en-gb/ RRID:SCR_016137

3.2. Methods

3.2.1. Primary rat oligodendroglial cell culture

3.2.1.1. PDL Coating of culture dishes

For the primary rat oligodendroglial precursor cell culture either 24-well plates (Greiner Bio-One) with glass coverslips (Hecht Assistent; 13 mm), 6-well plates (Greiner Bio-One) without coverslips or 96-well Black/Clear Flat Bottom TC-treated Imaging Microplates (Corning) were

used. 24-well plates were coated overnight at 4°C with 500 µl/well sterile filtered poly-D-lysine (PDL, Sigma) diluted in PBS (Sigma) for a final concentration of 0.25 mg/ml, to allow cell adhesion. 6-well plates were coated with 1500 µl/well of the same PDL solution overnight. 96-well plates were coated with 100 µl/well of 1 mg/ml PDL for 4 days. Plates were washed three times with PBS before addition of the cell suspension to avoid cytotoxic PDL remnants.

3.2.1.2. Preparation of rat oligodendroglial cell culture

Primary rat OPC cultures were generated as previously described (Göttle et al., 2015; Kremer et al., 2009; McCarthy & de Vellis, 1980). For this purpose, postnatal day 0 and 1 rats (Wistar rats of either sex) were anaesthetized with isoflurane (Piramal) for 3 minutes (min), disinfected with 70% ethanol (Merck) and sacrificed via decapitation. The brain was carefully detached from the skull, the lobules were then separated from each other with a spatula and cut with a scalpel. Following, meninges and blood vessels were carefully removed under the stereoscope. Afterwards, cortices were cut into small pieces and placed in MEM-Hepes medium (MEM, Thermo Fisher Scientific). Upon centrifugation at 2000 rpm for 1 min the pellet was resuspended in 1 ml digestion medium [MEM; 40 µg/ml DNAse; 0.24 mg/ml L-cystein; 30 U/ml papain (all Sigma-Aldrich)]. Following incubation at 37°C, 98% humidity, 5% CO_2 for 45 min, 1 ml of trypsin inhibitor solution [Leibvitz's medium (Thermo Fisher Scientific); 1 mg/ml trypsin inhibitor (Sigma-Aldrich); 50 mg/ml (BSA, Carl Roth); 40µg/ml DNase I type IV (Sigma-Aldrich)] was added and another incubation for 4 min at room temperature (RT) took place. Upon careful elimination of the supernatant, 1 additional ml of trypsin inhibitor solution was added to the pellet, followed by thorough mixing with an Eppendorf pipette (volume 1000 µl) and addition of 10 ml DMEM medium (Thermo Fisher Scientific) containing 10% FBS (Lonza/Capricorn). The solution was then centrifuged for 5 min at 1200 rpm and supernatant was discarded. In the next step, pellet was resuspended with a glass Pasteur pipette in 1 ml of mixed glial culture medium [DMEM; 10% FBS; 4 mM L-glutamine (Thermo Fisher Scientific); 100 U/ml penicillin/0.1 mg/ml streptomycin (Thermo Fisher Scientific)] and afterwards, another 14 ml of medium (for 6 rats) were added to the cell

suspension. Uncoated T-75 cell culture flasks (Greiner Bio-One) were equilibrated with 15 ml of culture medium before addition of 5 ml cell suspension (2 rats/flask). Cultures were incubated at 37°C, 98% humidity, 5% CO_2 for 10 days, medium was changed for the first time after 4 days and thereafter twice a week. This procedure resulted in a mixed glial culture containing an astrocytic layer, microglia and OPCs.

After 10 days, OPC purification was carried out according to the different properties of the diverse cell types on the surface of the culture flask. As a first step, flasks were shaken for 2 h at 180 rpm, at RT to achieve detachment and removal of debris and a part of the microglial cell population. Another 22 h of shaking were needed for the rest of microglia and OPCs to detach from the astrocytic layer. To separate OPCs from microglia, the supernatant of each flask was then placed on a 10 cm petri dish and incubated for 20 min at 37°C, 98% humidity, 5% CO_2 to attain the attachment of the microglia on the surface of the dish. The supernatant which consisted of ~98% OPCs was transferred from the dishes to a centrifuge tube and was centrifuged for 10 min at 1500 rpm. The pellet was then resuspended with a glass Pasteur pipette in 1 ml SATO medium [DMEM GlutaMax-I; 5 µg/ml bovine insulin; 50 µg/ml human transferrin; 100 µg/ml BSA (Carl Roth); 6.2 ng/ml progesterone; 16 µg/ml putrescine, 5 ng/ml sodium selenite; 400 ng/ml T3 (tri-iodo-thyronine); 400 ng/ml T4 (thyroxin; all Sigma-Aldrich); 4 mM L-glutamine; 100 U/ml penicillin/0.1 mg/l streptomycin (both Thermo Fisher Scientific)]. Cell number was counted under the light microscope using Thoma chambers (10 µl cell suspension and 10 µl trypan blue, Lonza) and the yield of the obtained cells was then calculated to 30000 cells/well (for 24-well plates), 2.4 x 10^6 (for 6-well plates) or 5000 cells/well (for 96-well plates). Upon seeding, cells were left to attach to the bottom of the wells for 1.5 h at 37°C, 98% humidity, 5% CO_2 in SATO medium (500, 3000 and 200 µl/well, respectively) and then spontaneous differentiation was induced via addition of differentiation medium (SATO; 0.5% FBS) in form of medium change. At the same time corresponding compound treatments were applied. Medium change was performed after 3 days.

3.2.1.3. Immunocytochemistry for the rat oligodendroglial cells.

For immunofluorescence staining cells were fixed with 4% paraformaldehyde (PFA, MERCK) in PBS. Cells in 24-well plates were washed 3 times before and after application of 500 µl PFA, and fixed for 10 min at RT. For fixation of cells in 96-well plates, half of the medium was removed and 8% PFA was carefully added on the surface to reach a final concentration of 4%. Fixation was performed for 15 min and was terminated via 3 washing steps with PBS. Plates were preserved at 4ºC.

Immunofluorescence staining was performed for marker expression analysis. Cells seeded on coverslips were fixed after 3 or 6 days of spontaneous differentiation and coverslips were placed on parafilm to stain for MBP on day 3 and MOG on day 6. Cells in 96-well plates were fixed after 1 day and stained for the p57kip2 protein directly in the plates. Unspecific staining was prevented everywhere by blocking with 10% normal goat serum (Sigma-Aldrich) for 45 min at RT. Staining procedures with primary rat anti-myelin basic protein (MBP, 1:250, Bio-Rad, Hercules, CA, USA Cat# MCA409S, RRID:AB_325004) were performed in blocking solution containing 0.02% Triton X-100 (Sigma-Aldrich) overnight at 4°C. Rabbit anti-p57kip2 primary antibody (Sigma-Aldrich Cat# P0357, RRID:AB_260850) was diluted 1:250 in blocking solution and applied overnight at 4°C. For staining with mouse anti-myelin oligodendrocyte glycoprotein antibody (MOG, 1:500, Merck Millipore, Cat# MAB5680, RRID:AB_1587278) cells were permeabilized with 0.1% Triton X-100 in blocking solution and 0.01% in antibody solution and the primary antibody was applied similarly overnight at 4°C. Upon 3 washing steps with PBS the secondary antibodies goat anti-rat Alexa Fluor 488 (1:500, Thermo Fisher Scientific Cat# A-11006, RRID:AB_2534074), goat anti-mouse Alexa Fluor 488 (1:500, Thermo Fisher Scientific Cat#A32728, RRID:AB_2633277) or goat anti-rabbit Alexa Fluor 594 (1:500, Thermo Fisher Scientific Cat# A-11037, RRID:AB_2534095) in PBS were applied for 2 h at RT. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:100, Roche), added in the secondary antibody solution. Finally, the coverslips were washed 3 times with PBS and mounted using Citifluor AF1 mountant solution (Electron Microscopy Sciences). Coverslips were fixed on microscopic slides (Engelbrecht)

with nail polish. Stained 96-well plates were also washed 3 times with PBS and after the last step 200 µl PBS/well were added for preservation of stained cells. Solutions were added at a volume of 90 µl/coverslip or 100 µl/well.

For image acquisition and analysis, the Zeiss Axionplan2 microscope (Carl Zeiss Microscopy, Jena, Germany) and the ImageJ BioVoxxel software (BioVoxxel Toolbox, RRID:SCR_015825) were used, respectively. Nine images per coverslip were captured in a Z like pattern using 20x magnification and the same exposure times throughout each experiment. Two coverslips were analyzed per condition. For quantification, the total cell number per field was assessed via DAPI staining. Subsequently, the number of protein marker-positive cells in relation to the total cell number was calculated and expressed as percentage. The analysis of the anti-p57kip2 staining will be described later in this section.

3.2.1.4. Chemical compound library screening

A phenotypic screening for compounds with the potential to promote p57kip2's protein nuclear exclusion was established. To this goal, the Prestwick Chemical Library (Prestwick) was chosen, and a total of 1280 small molecules were tested on primary rat OPCs, seeded onto PDL coated 96-well plates at a density of 5000 cells/well, as previously described. All compounds were applied at a concentration of 10 µM and the cells were allowed to differentiate for 24 h. The library was delivered at a concentration of 10 mM in DMSO, compounds were further diluted to 2 mM in DMSO and kept at -80°C until use. A maximum of 5 freeze-thaw cycles was performed for each plate. The compounds reached their final concentration upon a further intermediate 1/100 dilution in differentiation medium directly before the application onto the cells. Of note, to avoid cell detachment only half medium change was performed. Specifically, 100 µl SATO medium were removed and additional 100 µl differentiation medium with 2 times higher FBS and compound concentration were added (for overview of the consecutive dilutions see table below).

	Initial concentration	Dilution	Final concentration
1	10 mM	1/5 in DMSO	2 mM
2	2 mM	1/100 in differentiation medium	20 µM
3	20 µM	1/2 in differentiation medium	10 µM

The screening included application of 1 µM of the S1P agonist FTY720/Fingolimod [Echelon Biosciences; (Miron et al., 2008)] in triplicates on each plate as a positive control and differentiation medium +/- 0.5% DMSO as negative controls. Each compound was tested in triplicates within each individual experiment and each experiment was conducted twice. As previously described, the determination of the subcellular localization of the p57kip2 protein was accessed by means of immunofluorescence staining using the primary rabbit anti-p57kip2 antibody (p57kip2, 1:250, Sigma-Aldrich Cat# P0357, RRID:AB_260850) and the secondary goat anti-rabbit Alexa Fluor 594 antibody (1:500, Thermo Fisher Scientific Cat# A-11037, RRID:AB_2534095) in combination with DAPI.

Automated capturing of nine images per well with a 20x magnification was performed using the BD Pathway 855 High-Content Cell Analyzer (BD Biosciences). Data acquisition was performed with the Attovision v.1.7.1 software package (BD Biosciences). Briefly, the nuclear region of interest was defined according to the DAPI staining and the outer borders of the cytoplasmic according to the p57kip2 staining. Signal intensity of the p57kip2 staining was then automatically measured in each region of interest and data were exported as Excel files (Microsoft office). Analysis of the acquired data was conducted via an algorithm designed in house using the Matlab Software R2015a [Mathworks, Inc, RRID:SCR_001622; (Manousi et al., 2021); see box next page].

```
1   %This code calculates the percentage of cells with prevailed nuclear
2   %p57kip2 localization in one well, give that the p57kip2 signal intensity
3   %in the two regions (nucl vs. cyto) is provided as input.
4   %The only parameter that changes is the threshold nucl(line 22), which
5   %compensates for differences in the overall signal intensity among the
6   %plates. This threshold is being set upon manual analysis of the positive and
7   %negative controls and under our experimental conditions was always 1000+/-
8   %200.
9   %Note: this threshold has to be set for each plate and is strictly being kept
10  %constant for the whole plate.
11  A=xlsread('excel file name','excel sheet name','cytoplasmic p57 intensity values:nuclear p57 intensity values');
12  cyto=A(:,1);
13  nucl=A(:,2);
14  r=cyto./nucl;
15  p=(numel(r));
16  j=0;
17  k=0;
18  while j<=1000;
19      if r>=1.3;
20         j=j+1;
21         break
22      else and(r<1.3,nucl>1100);
23          k=j+0;
24          break
25      end
26  end
27  w=double(ans);
28  s=sum(ans,1);
29  l=(s/p)*100;
30     fprintf('%% cells with nuclear p57kip2: %f\n',l)
```

The values of the p57kp2 signal intensity in the two regions of interest separately were provided as input. The output of the analysis was the percentage of cells with prevailed nuclear p57kip2 localization (1.3 times more) in each well. Following, the mean of the triplicates for each tested compound was calculated and compared to a specific threshold. As hit compounds, were chosen those which induced equal or greater than 0.75 fold change in nuclear localization of the p57kip2 protein in comparison to the negative controls. This threshold was chosen based on previous experiments in our research group and corresponds to the maximum increase in nuclear exclusion between days 1 and 3 upon initiation of spontaneous differentiation that has been detected. This time window is considered critical since it represents the time when significant transition of OPCs to oligodendrocytes takes places (Göttle et al., 2015). This threshold was kept constant throughout the screening. Since that staining was performed in a non-automated way and the overall intensity was varying among the experiments, an additional threshold was used to

compensate for variations in the signal intensity of the staining among the several plates and was adapted in every run accordingly. To this end, positive and negative controls were used as means of calibration.

3.2.2. Human fetal oligodendroglial cell culture

3.2.2.1. Preparation of human fetal oligodendroglial cell culture

These experiments were conducted by our collaborators in the Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC H4A 3K9, Canada and were published as part of the study from Manousi et al., 2021. The culture was prepared from human fetal second-trimester (14-17 weeks) telencephalon tissue samples which were collected from elective abortions and provided by the University of Washington Birth Defects Research Laboratory, Seattle, Washington, USA, within an NIH supported program (MP-37-2014-540; 13-244-PED; eReviews_3345). Tissue digestion was performed with 0.25% trypsin (Thermo Fisher Scientific) and 25 µg/mL DNase I (Roche) to obtain dissociated cells. Isolation of OPCs, that comprised only ~0.1% of the total cells ware conducted via Magnetic-activated cell sorting using immunomagnetic beads coated with O4 antibody (Miltenyi Biotec, Cat# 130-096-670, RRID:AB_2847907). This experimental procedure is well established by our collaborators and previously published elsewhere (Cui et al., 2012; Cui et al., 2010; Leong et al., 2014). Isolated OPCs were then plated in 96-wells plates coated with poly-L-lysine (Sigma-Aldrich) and extracellular matrix (ECM-gel from Engelbreth-Holm-Swarm murine sarcoma, Sigma-Aldrich) at a density of 10000 cells/well. Cells were cultured in DMEM-F12 media supplemented with N1, B27 supplement, 10 ng/ml PDGF-AA and 10 ng/ml bFGF (all Sigma-Aldrich) for 4 days before the initiation of the compound treatment. Compounds were tested as triplicates for 4 and 6 days. Medium change was performed every 2 days.

3.2.2.2. Immunocytochemistry for the human fetal oligodendroglial cells

Cells were live stained with application of primary mouse anti-O4 (1:200, R&D Systems Cat# MAB1326, RRID:AB_357617), and hybridoma anti-GalC IgG3 (GC, 1:50, Montreal

Neurological Institute, McGill University, Montreal, Quebec, Canada) monoclonal antibodies for 15 min at 37ºC and then fixed with 4% PFA for 10 min in RT. Secondary antibodies goat anti-mouse IgM-Alexa Fluor 647 (1:500, SouthernBiotech Cat# 1021-31, RRID:AB_2794254) to O4 and goat anti-mouse IgG3-Alexa Fluor 488 (1:500, Thermo Fisher Scientific Cat# A-21151, RRID:AB_2535784) to GC were applied for 30 min at RT. Cell nuclei were stained with Hoechst 33258 (1:1000, Thermo Fisher Scientific Cat# H3569, RRID:AB_2651133). Following cell imaging, the O4- and GC-positive cells were counted, and their percentage was calculated in two independent preparations from human donor tissues (HF601 and HF603).

3.2.3. Western blotting

These experiments were conducted in collaboration with Laura Reiche and Dr. Jessica Schira-Heinen. For western blot analysis, OPCs were seeded at a density of 2.4 x 10^6 cells/well in PDL coated 6-well plates and after incubation in Sato medium for 1.5 h, cells were treated either with 0.1 µM parbendazole or with the corresponding DMSO concentration for control in differentiation medium for 1 h. Cell detachment was achieved with removal of the medium, 3 washing steps with 1.5 ml ice cold PBS and incubation with 1.5 ml pre-warmed trypsin-EDTA (Capricorn Scientific; diluted 1:1 with PBS) for 3 min at 37ºC, 98% humidity, 5% CO_2. Following incubation, the enzymatic reaction was stopped with differentiation medium. Cells were then scraped thoroughly; surface was washed once with 1.5 ml PBS and all supernatants were collected in a centrifuge tube. In the next step, cells were centrifuged at 2100 rpm for 10 min at 4°C, supernatant was discarded, and pellets were frozen on dry ice prior to storage at -80°C.

Cell lysis was carried out on ice with approximately 60 µl 1x radioimmunoprecipitation assay buffer (RIPA buffer, Cell Signaling Technology, Danvers, MA, USA) pro sample, supplemented with HALT™ Protease-/Phosphatase inhibitor cocktail and EDTA (both Thermo Fisher Scientific; 1:100). Upon pellet resuspension in lysis buffer, samples were subjected to 10 seconds (sec) of sonication with an ultrasound homogenizer

(SonopulsHD2070, 50% power, pulse 0.5 sec on and 0.5 sec off) and subsequently centrifuged at 14000 rpm for 10 min at 4°C, to proceed with the supernatant.

For determination of the protein concentration 5 ml of the supernatant were diluted 1:3 in deionized water. Similarly, protein standard series (BSA-stocks: 0 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml) were thawed and diluted 1:3 in water. The next steps were performed according to the instructions of the manufacturer of the DC™ Protein Assay Kit II (Bio-Rad). Each sample and standard were measured in duplicate. For each replicate 25 µl Reagent A and 0.5 µl of Reagent S were used. First 5 µl of samples or standards were transferred to a 96-round well plate (Corning), followed by addition of a mix of A and S reagents and 200 µl of Reagent B. Plates were immediately covered with aluminum foil and protein quantification with the Infinite M200 Pro plate reader (TECAN) was performed within the next 10 min (for the main settings see the table below).

Parameter	Setting
Mode	Absorption
Multiple reads per well Square (filled)	3 x 3
Multiple reads per well (Border)	2000 µm
Wavelength	540 nm
Bandwidth	9 nm
Number of flashes	25
Settle Time	0 ms

Samples were then subjected to standard sodium dodecyl sulfate (SDS) gel electrophoresis using Bolt 12% Bis-Tris Plus mini protein gel (Thermo Fisher Scientific). Pro lane 20 µg sample were loaded along with the Bolt LDS Sample Buffer (4x), Bolt Reducing Agent (10x) (both Thermo Fisher Scientific) and deionized water as shown in the table below.

Reagent	Reduced Sample
Sample	x µL
Bolt LDS Sample Buffer (4x)	10 µL
Bolt Reducing Agent (10x)	4 µl
Deionized water	to 26 µL

Total Volume	40 µL

Samples were incubated at 70ºC for 10 min and spun down before use. The Bolt MOPS SDS Run Buffer (20x) was diluted to 1x with deionized water and added to the chamber of the XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific). Upon short rinsing of the gel with deionized water and placement in the chambers, samples along with 4 µl Magic Marker (Thermo Fisher Scientific) and 8 µl SeeBlue™ Pre-stained Protein Standard (Thermo Fisher Scientific) were loaded to the wells. Gels run at constant voltage, 15 min at 50 V, afterwards 200 V for around 40 min. The run was paused 10 min before it was finished to load 5 µl Pierce™ Lane Marker Non-Reducing Sample Buffer in each well and then it was restarted to the end.

For the semi-dry western blotting, first the Pierce™ 1-Step Transfer Buffer (Thermo Fisher Scientific) was diluted 1:5 in distilled water. In total 50 ml were required per gel. The nitrocellulose membrane and the filters (Thermo Fisher Scientific) were equilibrated in pre-diluted 1-Step buffer for 5 min. First two filter papers were placed in the center of the bottom part of the cassette (anode) and then the membrane. The size of the gel was corrected and upon immerging in distilled water for 5-15 sec to facilitate easy positioning, it was placed on the top of the membrane. Two more pre-wetted filter papers were placed on top of the gel and the cathode lid (top of cassette) was placed on the top. Of note, the efficiency of the transfer is heavily dependent on the proper removal of air bubbles after the addition of every layer. Power Blotter–Semi-dry Transfer System run using the settings presented in the table below.

Methods	Constant	Default Run Time [min]
Low MW (< 25 kDa)	1.3 Amps	5
Mixed-Range MW (25-150 kDa)	1.3 Amps	7
High MW (>150 kDa)	1.3 Amps	10
Std Semi Dry	25 Volts	60
1.5 mm thick gels or unknown gel size	1.3 Amps	10

For evaluation of the efficiency of protein transfer following SDS electrophoresis the Pierce Reversible Protein Stain Kit (Thermo Fisher Scientific) was used. For staining, the membrane was quickly rinsed with ultrapure water and 15 ml of the MemCode Reversible Protein Stain was added to the membrane. Upon incubation for 30 sec on a rotary platform shaker, stained proteins appeared as turquoise-blue bands. Background staining was then removed with 3 subsequent steps of washing with 15 ml of the MemCode Destain Reagent and incubation for 5 min in the same reagent on a rotary platform shaker. Following, the membrane was washed 4 times with ultrapure water and was incubated in water for 5 min on a rotary platform shaker. Photos were taken using the Fusion FX device (Vilber Lourmat) to detect the total amount of loaded protein. Afterwards, staining was removed by incubation in 20 ml MemCod Stain Eraser for 2 min on a rotary platform shaker and 4 washing steps with ultrapure water followed by incubation for 5 min on a rotary platform shaker.

For specific protein detection first unspecific binding was blocked with 1% skimmed milk powder (Carl Roth) in 20 ml TBS (20 mM Tris base, 150 mM NaCl ad 1000 ml ddH2O; pH adjusted to 7.6 using HCl) per membrane for 1 h on a rotary shaker at RT. Application of the following primary antibodies: rabbit anti-p38 (1:1000, Cell Signaling Technology Cat# 9212, RRID:AB_330713), rabbit anti-p-p38 (1:1000, Cell Signaling Technology Cat# 9211, RRID:AB_331641), mouse anti-GAPDH (1:5000, Merck Millipore Cat# MAB374, RRID:AB_2107445), was performed in 10 ml TBS Tween solution [TBST, 1x TBS; 0.05 % (v/v) Tween 20] implemented with 1% skimmed milk, overnight on a rotary shaker at 4°C. The next day, membrane was washed 3 times with TBST for 10 min on a rotary shaker each and following the secondary antibodies anti-rabbit IgG, HRP-linked (1:2000, Cell Signaling Technology Cat# 7074, RRID:AB_2099233) and anti-mouse IgG (H+L), made in horse (1:5000, Vector Laboratories, Burlingame, CA, USA Cat# PI-2000, RRID:AB_2336177) were diluted in 10 ml TBST and applied to the membrane for 1 h on a rotary shaker at RT. Signals were visualized using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) applied for 5 min. Membranes were first used to detect p-p38 and afterwards stripped for the detection of p38 and the housekeeping protein (GAPDH), all in a sequential

manner on the same membrane to ensure reliable quantification (see chapter 8.3). Stripping was performed with incubation of the membrane with 10 ml ReBlot Plus Strong Solution (10x, Merck Millipore) diluted 1:10 with distilled water for 15 min at RT on the shaker. Finally, membrane was washed with TBST for 10 min and afterwards two 2 times with TBS for 5 min each. Protein bands were quantified using the Fusion FX software (Vilber Lourmat). The intensity for the bands was determined and normalized to the total amount of the loaded protein, as well as the intensity of the GAPDH band of the corresponding sample.

3.2.4. Organotypic cultures of cerebellar slices

3.2.4.1. Preparation of organotypic cultures of cerebellar slices

Preparation of organotypic cultures of cerebellar slices was performed according to an established protocol (Birgbauer et al., 2004; Jadasz et al., 2018) with the addition of a few adaptations. For this purpose, P7 Wistar rats of both sexes were anesthetized with isoflurane for 3 min, disinfected with 70% ethanol and sacrificed via decapitation. The whole brain was carefully detached from the skull and placed in a petri dish filled with ice cold Ringer solution (Fresenius Kabi) implemented with 1 mM $MgCl_2 \cdot 6H_2O$ (SERVA), 5 mM HEPES (Thermo Fisher Scientific), pH: 7.2, where the cerebellum was separated from the rest of the brain using a scalpel. The intact cerebellum was then embedded in 4% agarose (UltraPure Low Melting Point; Thermo Fisher Scientific; diluted in Ringer solution) and mounted vertically to the flat metal chuck of the HM 650V microtome (Thermo Fisher Scientific) using a drop of superglue (UHU). Afterwards, the metal chuck with the attached cerebellum was placed in a magnetic buffer tray which was filled with ice cold Ringer solution. Sagittal sections, 350 µm thick, were made using the following settings: cutting frequency: 50 Hz, cutting amplitude: 1 mm, cutting speed: 12 mm/s and plated onto cell culture inserts (hydrophilic PTFE, pore size: 0.4 µm, diameter 30 mm; Merck Millipore) in 6-well plates upon addition of 1 ml of culture medium [50% MEM, 25% HBSS +/+, 25% heat inactivated horse serum; containing 100 U/ml penicillin/0.1 mg/ml streptomycin (all Thermo Fisher Scientific), 5 mg/ml D-glucose monohydrate (Merck Millipore)] underneath the membrane. Of note, the culturing plates are prepared at least 1 h before the initiation of the sectioning and inserts were equilibrated with

addition of 1 ml culture medium on their top and incubation at 37°C, 98% humidity, 5% CO_2. The top side of the insert was carefully dried via aspiration before plating to allow attachment of the slices. Compounds were applied 4 h later as medium change. From a single animal 6 to 8 cerebellar slices were obtained, plated onto 2 inserts, and kept in culture for 3 to 7 days (37°C, 98% humidity, 5% CO_2). For each individual experiment slices from one animal were used to test one compound and the corresponding DMSO control.

3.2.4.2. Immunohistochemistry for organotypic cultures of cerebellar slices

Prior to fixation slices were washed once with 1 ml PBS both on the top and underneath the insert. They were fixed with 4% PFA for 20 min at RT and then washed 3 times in the same fashion with PBS. Cultures were preserved in PBS at 4°C until the initiation of the immunofluorescence staining. Staining procedure was performed free-floating with the slices being still attached to the inserts. To do so the central part of the insert was separated from the outer plastic ring and placed in a well of a 12-well plate followed by addition of PBS. Each solution was added at a volume of 500 µl. Initially, permeabilization of cells was performed with 0.5% Triton X-100 in PBS and non-specific binding was prevented by incubation in blocking solution [10% NGS (Sigma-Aldrich), 1% BSA (Thermo Fisher Scientific), 0.2% Triton X-100 in PBS]. Primary antibodies rabbit anti-neurofilament (NF, 1:1000, Abcam, Cat# ab8135, RRID:AB_306298), rat anti-MBP (1:250, Bio-Rad Cat# MCA409S, RRID:AB_325004) and mouse anti-OLIG2 (OLIG2, 1:500, Merck Millipore Cat# MABN50, RRID:AB_10807410) were diluted in primary antibody solution [10% NGS, 1% BSA, 0.1% Triton X-100 in PBS]. Secondary antibodies goat anti-rabbit Alexa Fluor 405 (1:500, Thermo Fisher Scientific Cat# A-31556, RRID:AB_221605), goat anti-rat Alexa Fluor 488 (1:500, Thermo Fisher Scientific Cat# A-11006, RRID:AB_2534074) and goat anti-mouse Alexa Fluor 647 (1:500, Thermo Fisher Scientific Cat# A32728, RRID:AB_2633277) were diluted in secondary antibody solution (1% NGS, 1% BSA, 0.1% Triton X-100 in PBS). All solutions were incubated overnight at 4°C and slices were finally mounted upwards on a microscopic slide (Engelbrecht) using Citifluor and covered with a square-shaped glass which was fixed

with a few drops of nail polish. Image acquisition was performed with a confocal microscope (CLSM 510, Carl Zeiss Microscopy) and analyzed with the Zen 2 Blue Edition (Carl ZEISS Microscopy, ZEN Digital Imaging for Light Microscopy, RRID:SCR_013672) and ImageJ BioVoxxel software packages. In total 3 images (40x magnification) per field were obtained via Z-stack scanning covering 2 µm depth in total and prior the analysis they were projected onto a single plane via orthogonal projection. The orthogonal projection enabled a better tracking of the MBP-positive cell processes and the detection of the characteristic structure of a myelinating MBP/OLIG2-positive cell which was defined as criterion for the analysis (Fig. 10a-a'''). To assess the number of myelinating oligodendrocytes in relation to the total number of OLIG2-positive cells 12-16 fields from similar structures were analyzed for all conditions.

3.2.5. Cuprizone-mediated demyelination and remyelination mouse model

All *in vivo* experiments were performed with ethical care in the animal facility of the Heinrich-Heine-University (Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben; ZETT) under pathogen-free conditions. Seven-week-old female C57BL/6 mice (Janvier Labs) delivered to the animal facility, were separated into groups of 2 or 3 and left for 1 week to get acquainted to the environment before the initiation of the experiment. During this time animals were fed standard rodent chow (SSNIFF, Cat# V1534). As inclusion criteria same age and body weight between 17-19 gr were used. The software G*Power was used to compute a required size of six animals per group with effect size d 2.6 and confidence interval 95%. Demyelination was induced by feeding animals with 0.2% (w/w) cuprizone [bis(cyclohexanone)oxaldihydrazone]-containing diet [either from Envigo (Cat# TD.140803) or from SSNIFF Spezialdiäten GmbH (maintenance diet pellets 10 mm, V1534 implemented with 0.2% cuprizone, Sigma-Aldrich, CAS 370-81-0) for 6 weeks. Spontaneous remyelination was initiated thereafter upon switch to the standard rodent chow.

Parbendazole (MedChem Express, CAS 14255-87-9) was administered at a concentration 1.14 mg/ml and using 20 mg/kg body weight as dose. Parbendazole stock concentration of

13.33 mg/ml in DMSO was further diluted using sterile saline solution (Fresenius Kabi) implemented with 2% Tween 80 (Sigma-Aldrich). A total volume of 350 µl of the parbendazole solution or the corresponding vehicle solution (8.6% DMSO in sterile saline solution implemented with 2% Tween 80) was administered daily via intraperitoneal (i.p.) injections for the last 17 days of cuprizone treatment. Preparation of the solution was performed under sterile conditions and the injection was prepared in the animal facility to avoid contamination with external pathogens. Injections were performed using 1 ml syringes (Braun) and 0.45 x 10 mm needles (BD Microlance 3). Danazol (Sigma-Aldrich, CAS 17230-88-5) was initially dissolved in DMSO for a stock concentration of 30 mg/ml and was diluted before use 1:1 in Tween 80 to increase stability in aqueous solutions. Preparation of the solution was performed under sterile conditions and only after entering the animal facility, it was further diluted down to a final concentration of 0.4 mg/ml in drinking water from the same source as always to avoid contamination with external pathogens. Every day 5 ml of fresh solution was prepared per animal to perform oral administration of danazol at a dose of 100 mg/kg body weight. Vehicle-treated animals were administered the same amount of water implemented with 1.3% DMSO and 1.3% Tween 80. Body weight of the animals was controlled twice a week throughout the experiments. A decrease of more than 10% would be indication of suffering and reason for immediate sacrifice of the animal. One week upon cuprizone withdrawal, treated and naïve animals of the same age were deeply anesthetized using isoflurane inhalation for 2 min and transcardially perfused with 20 ml PBS followed by 20 ml 4% PFA in PBS. Unresponsive mice to toe/tail pinches were fixed in a supine position (lying on their back) by taping the forepaws to a surface made of Styrofoam inside a chemical fume hood. An incision through the skin with surgical scissors along the thoracic midline from beneath the xiphoid process up to the clavicle was made and was followed by two additional from the xiphoid process along the base of the ventral ribcage laterally. Following exposure of the thoracic field, the thoracic musculature and ribcage between the breastbone and medial rib insertion points were cut and incision was extended up to the clavicles. Thereafter, the pericardial sac was completely torn and the skin underneath the

neck along with the ribcage were pinned upwards to expose the heart. Connective tissue around the heart was removed. The beating heart was then fixed with blunt forceps, a butterfly needle (BD Vacutainer) was inserted to the left ventricle, and a small incision in the right atrium was made. The needle was kept fixed throughout the process with the fingers. Upon perfusion, animals were decapitated, and brains were then removed and post-fixed overnight in 4% PFA (3 ml) at 4°C on a rotary shaker. For an overview of the groups of animals included in this study see table below.

Age (weeks)/ sex	**CPZ (weeks)**	**Remyelination (weeks)**	**No of animals**	**Treatment/duration (days)**
8/female	6	1	5	Parbendazole (20 mg/kg); i.p.; 350 µl/ 17
8/female	6	1	5	Vehicle Par. (8.6% DMSO in sterile saline solution with 2% Tween 80); i.p. 350 µl/ 17
8/female	6	1	5	Danazol (100 mg/kg); oral/ 17
8/female	6	1	5	Vehicle Dan. (1.3% DMSO in drinking water with 1.3% Tween 80)
15/female	-	-	6	Naive

Following post-fixation, cryoprotection of mouse brains was performed in 30% sucrose (Carl Roth; in PBS) at 4°C for 48 h on a rotary shaker. Brains were then embedded in Tissue-Tek OCT medium (Sakura Finetek), frozen in ice cold 2-Methylbutane (Merck) and stored at -80°C until sectioning with cryostat (Leica CM3050S). For the sectioning SuperFrost Plus microscope slides (Thermo Fisher Scientific) were used. A total of 40 coronal 10 µm sections per region of interest were prepared and stored at -80°C. Regions of interest, medial (Bregma -0.82 to -1.22 mm) and caudal (Bregma -1.94 to -2.34 mm) parts of corpus callosum, were defined according to (Franklin & Paxinos, 2008).

For immunohistochemical staining, sections were first thawed and air-dried for 10 min at RT. Rehydration was performed for 5 min in distilled water, followed by transfer to -20°C acetone (Merck) for 5 min. Afterwards, 2 washing steps first in 1x TBS (20x TBS: 122 gr TRIS, 180 gr NaCl at 1000ml H_2O; pH 7.6) and then 1x TBS-T (TBS implemented with 0.02% Triton X-

100; pH 7.6) for 5 min each were performed. A waterproof ring was drawn around each section using a Mini Pap Pen (Thermo Fisher Scientific) to allow application of the solutions (approximately 60 µl per section). Non-specific staining was blocked with 10% biotin-free bovine serum albumin (BSA, Carl Roth) in TBS-T or 10% normal donkey serum (NDS, Sigma-Aldrich) in TBS-T for 30 min at RT. Primary antibody solution (10% BSA in 1x TBS or 10% NDS in 1x TBS) was then applied and incubated overnight at 4°C. The following primary antibodies were used: mouse anti-APC (CC1, 1:500, GeneTex Cat# GTX16794, RRID:AB_422404); rabbit anti-glutathione-S-transferase pi (GSTpi, 1:2000, Enzo Life Sciences, Cat# ADI-MSA-101-E, RRID:AB_2039147); goat anti-PDGFR (alpha/CD140A, 1:250, Neuromics, Cat# GT15150, RRID:AB_2737233); rat anti-PLP [PLP, 1:250, kind gift from B. Trapp and R. Dutta, Dept. of Neurosciences, Cleveland Clinic, OH, USA (Chen et al., 2015)]. Slices were then washed twice in 1x TBS for 5 min each. Secondary antibodies were diluted 1:200 and applied for 30 min along with DAPI (1:50) in PBS at RT. The following secondary antibodies were used: goat anti-rabbit Alexa Fluor 594 (1:200, Thermo Fisher Scientific Cat# A-11037, RRID:AB_2534095); goat anti-rat Alexa Fluor 488 (1:200, Thermo Fisher Scientific Cat# A-11006, RRID:AB_2534074); goat anti-rabbit Alexa Fluor 488 (1:200, Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165); goat anti-mouse Alexa Fluor 488 (1:200, Thermo Fisher Scientific Cat# A32728, RRID:AB_2633277); donkey anti-goat Alexa Fluor 488 (1:200, Thermo Fisher Scientific Cat# A-11055, RRID:AB_2534102). Two final washing steps were performed with 1x TBS-T and 1x TBS for 5 min each and the sections were mounted with Shandon™ Immu-Mount (Thermo Fisher Scientific). For image acquisition and analysis, a CLSM and the Zen 2 Blue edition and ImageJ BioVoxxel software packages were used, respectively. Photos were taken with a 20x magnification along the midline of the corpus callosum. For each mouse 4 coronal sections were stained and analyzed (2 from the medial and 2 from the caudal region of interest).

3.2.6. Statistical analysis

Graph design and statistical analyses were performed using the GraphPad Prism 8.0.2 software (GraphPad Prism, San Diego, CA, RRID:SCR_002798). The absence of Gaussian

distribution of the data was assessed via Shapiro-Wilk normality test. Statistical significance between two groups was assessed via unpaired Mann-Whitney U test and among three groups with Tukey's range test following one-way ANOVA. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n represents the number of independent experiments. The G*Power 3.1.9.2 software (Faul et al., 2009) and the Wilcoxon-Mann-Whitney test (two groups) were used to compute *a priori* sample size for the *in vivo* experiments.

4. RESULTS

4.1. Phenotypic screening for compounds that promote p57kip2's nucleocytoplasmic shuttling[1]

Previous data from our research group indicate that nuclear exclusion of the p57kip2 protein is an early event during the course of spontaneous oligodendroglial cell differentiation and that altered gene expression or nucleocytoplasmic shuttling dynamics have an impact on the process (Göttle et al., 2015; Kremer et al., 2009). Based on this information a phenotypic compound screening using p57kip2's protein subcellular localization as readout to identify pharmacological compounds with the potential to positively regulate already early stages of oligodendrogenesis (Fig. 5a) was established and performed (Manousi et al., 2021). For this purpose, the Prestwick Chemical Library, a widely used collection of mostly FDA/MDA (US Food and Drug Administration/Europeans Medicines Agency) approved small molecules was chosen and tested on primary OPC cultures. All compounds were applied in triplicates at a concentration of 10 µM and upon 24 h of spontaneous differentiation, cells were fixated and stained for the p57kip2 protein. The application of positive (1 µM Fingolimod) and negative (differentiation medium with or without 0.5% DMSO) controls (Fig. 5c-c'') on each 96-well plate worked as means of calibration compensating for differences in the intensity of each individual staining and all the experimental procedures were performed by the same person under the same conditions to increase efficiency of the screening. Image acquisition for determination of the subcellular localization of the protein was performed automatically using the BD Pathway 855 High-Content Cell Analyzer. Measurements and analysis of the signal intensity were performed as well in an automated manner using the Attovision v.1.7.1 software and an algorithm designed in house, respectively. As threshold for hit consideration was used a ≥ 0.75 fold change in nuclear localization of the protein in comparison to the negative controls. For the primary hit selection each experiment was repeated twice and for confirmation all primary hits were tested three more times using the same experimental set

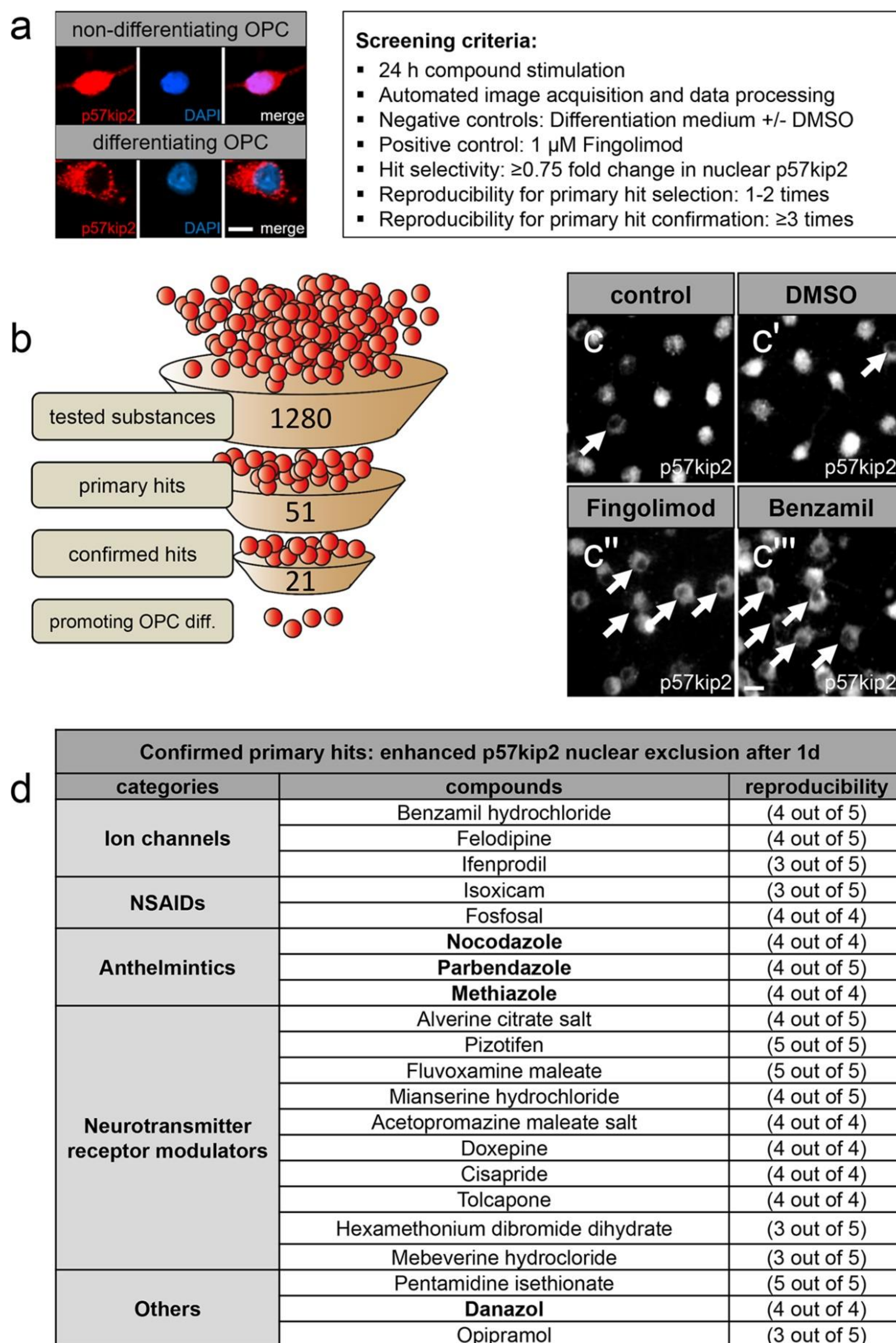
[1] Parts of these results are published in Manousi et al, 2021; see chapter 8.1

up. Only effects detected at least three times in total (out of five) were considered sufficient for hit compound determination.

Primary hits: enhanced p57kip2 nuclear exclusion after 1 day	
1	Mebeverine hydrochloride
2	Ifenprodil tartrate
3	Halcinonide
4	Benzamil hydrochloride
5	Opipramol dihydrochloride
6	Biotin
7	Felodipine
8	Alverine citrate salt
9	Methocarbamol
10	Hexamethonium dibromide dihydrate
11	Diflunisal
12	Isoxicam
13	Sulfamethoxypyridazin
14	Azaguanine-8
15	Ethambutol dihydrochloride
16	Propafenone hydrochloride
17	Norcyclobenzaprine
18	Pentamidine isethionate
19	Methiazole
20	(+,-)-Synephrine
21	Lansoprazole
22	Leflunomide
23	Denatonium benzoate
24	Norgestimate
25	Pizotifen malate
26	Fluvoxamine maleate
27	Acyclovir hydrochloride
28	Mianserine hydrochloride
29	Cilnidipine
30	Danazol
31	Aminohippuric acid
32	Acetopromazine maleate salt
33	Furaltadone
34	Guaifenesin
35	Bendroflumethiazide
36	Propidium iodide
37	Methotrimeprazine maleat salt
38	Fosfosal
39	Tolcapone
40	Nocodazole
41	Doxepin hydrochloride
42	Clomipramine hydrochloride
43	Vincamine
44	Ketoconazole
45	Fusidic acid sodium salt
46	Cisapride
47	Thiorphan
48	Oxybenzone
49	Hymecromone
50	Methyldopate hydrochloride
51	Parbendazole

The screening has been successfully completed with a total number of 1280 tested compounds and out of these, 51 primary hits were identified (for the complete list see table above). Upon confirmation 21 substances (Fig. 5d) were found to reproducibly enhance p57kip2 translocation to the nucleus (as shown for benzamil hydrochloride in Fig. 5c'''). In addition, doxorubicin was found to induce exclusive nuclear localization of the p57kip2

protein (data not shown). The results of the three validation rounds are presented in Annex; paragraph 8.4.1. Although, the evaluation of the statistical data quality of the screening was not satisfactory [Z factor<0; (Zhang et al., 1999)] hit overlap with other screenings for compounds with promyelinating properties, namely ifenprodil and isoxicam, was detected. Furthermore, some of the identified hits are related to pathways that have previously been described to regulate oligodendroglial cell lineage progression and survival, as for example the histamine receptor-3 (H3R) inverse agonist GSK247246 (Chen et al., 2017) and benztropine which targets muscarinic receptors (Deshmukh et al., 2013), thus proving the biological significance and efficiency of the screening.



Confirmed primary hits: enhanced p57kip2 nuclear exclusion after 1d		
categories	**compounds**	**reproducibility**
Ion channels	Benzamil hydrochloride	(4 out of 5)
	Felodipine	(4 out of 5)
	Ifenprodil	(3 out of 5)
NSAIDs	Isoxicam	(3 out of 5)
	Fosfosal	(4 out of 4)
Anthelmintics	**Nocodazole**	(4 out of 4)
	Parbendazole	(4 out of 5)
	Methiazole	(4 out of 4)
Neurotransmitter receptor modulators	Alverine citrate salt	(4 out of 5)
	Pizotifen	(5 out of 5)
	Fluvoxamine maleate	(5 out of 5)
	Mianserine hydrochloride	(4 out of 5)
	Acetopromazine maleate salt	(4 out of 4)
	Doxepine	(4 out of 4)
	Cisapride	(4 out of 4)
	Tolcapone	(4 out of 4)
	Hexamethonium dibromide dihydrate	(3 out of 5)
	Mebeverine hydrocloride	(3 out of 5)
Others	Pentamidine isethionate	(5 out of 5)
	Danazol	(4 out of 4)
	Opipramol	(3 out of 5)

Figure 5. ***In vitro* phenotypic screening led to the identification of compounds that promote nuclear exclusion of the p57kip2 protein in primary rat OPCs.** Presentation of the readout and the experimental setup of the primary screening procedure (a). Funnel illustration showing the progression of the screening (b). Representative images taken using the BD Pathway 855 High-Content Cell Analyzer visualizing p57kip2's subcellular localization upon treatment with the negative controls, differentiation medium without (control) and with 0.5% DMSO, the positive control Fingolimod (1µM)

and a prominent primary hit benzamil hydrochloride (c-c'''). Image magnification: 10x. List of the confirmed primary hits, assigned to different categories according to their biological significance (d). Arrows in c-c''' indicate at cells with cytoplasmic p57kip2 localization. Scale bars: 10µm. Figure 1 from (**Manousi et al., 2021**) Anastasia, M., Peter, G., Laura, R., Qiao-Ling, C., Luke, M. H., Rainer, A., Joel, G., Jessica, S.-H., Jack, P. A., Hans-Peter, H., & Patrick, K. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/https://doi.org/10.1016/j.ebiom.2021.103276.

4.2. Secondary screening resulted in identification of compounds that promote OPC differentiation *in vitro*[2]

The first step for the assessment of myelin repair properties was to screen the primary hit compounds for their capacity to promote transition from OPCs to mature myelinating oligodendrocytes. To this goal, rat primary OPCs were cultured and stimulated with compound concentrations ranging from 10 to 0.001 µM. Cells were allowed to differentiate for three days upon compound stimulation and the expression of the late protein myelin marker MBP was detected. Potential cytotoxic effects were accessed via evaluation of total cell numbers (DAPI counts, data not shown). This secondary screening led to the identification of four substances with the capacity to positively modulate OPC differentiation both at early and late stages and the most efficient and well-tolerated concentrations were determined [Fig. 6; (Manousi et al., 2021)].

Stimulation with 0.02 µM parbendazole, 0.1 µM methiazole or 5 µM danazol significantly increased the number of MBP-positive oligodendrocytes (Fig. 6m-o). Treatment with 0.1 µM nocodazole led to a non-significant induction of MBP-positivity, however reproducibly resulted in more complex morphological phenotypes (Fig. 6i-i', p). The absence of significance of the nocodazole induced effects is attributed to cytotoxicity, which was revealed as a 31.43% decrease of the total cell number (DAPI counts). The compounds which were found to significantly induce oligodendrogenesis were additionally evaluated upon 6 days of application, using as myelin marker the MOG protein, to further assess their differentiation promoting effects and their tolerability. Cells treated with 0.02 µM

[2] Parts of these results are published in Manousi et al, 2021; see chapter 8.1

parbendazole for six days were characterized by increased MOG-positivity (Fig. 2s). Methiazole and danazol induced similar effects in cellular maturation at this stage, however to a lower extent (Fig. 6q-r). Of note, treatment with the benzimidazole derivatives, parbendazole and methiazole not only increased the percentage of MOG-positivity but also led to enhanced morphological maturation of the cells (Fig. 6k-l'). In addition, no significant differences in the cell counts were detected suggesting good tolerability of the tested compounds. These experiments suggest that p57kip2's subcellular localization can efficiently be used as an early OPC marker which can successfully lead to identification of compounds promoting both early and late stages of cell differentiation.

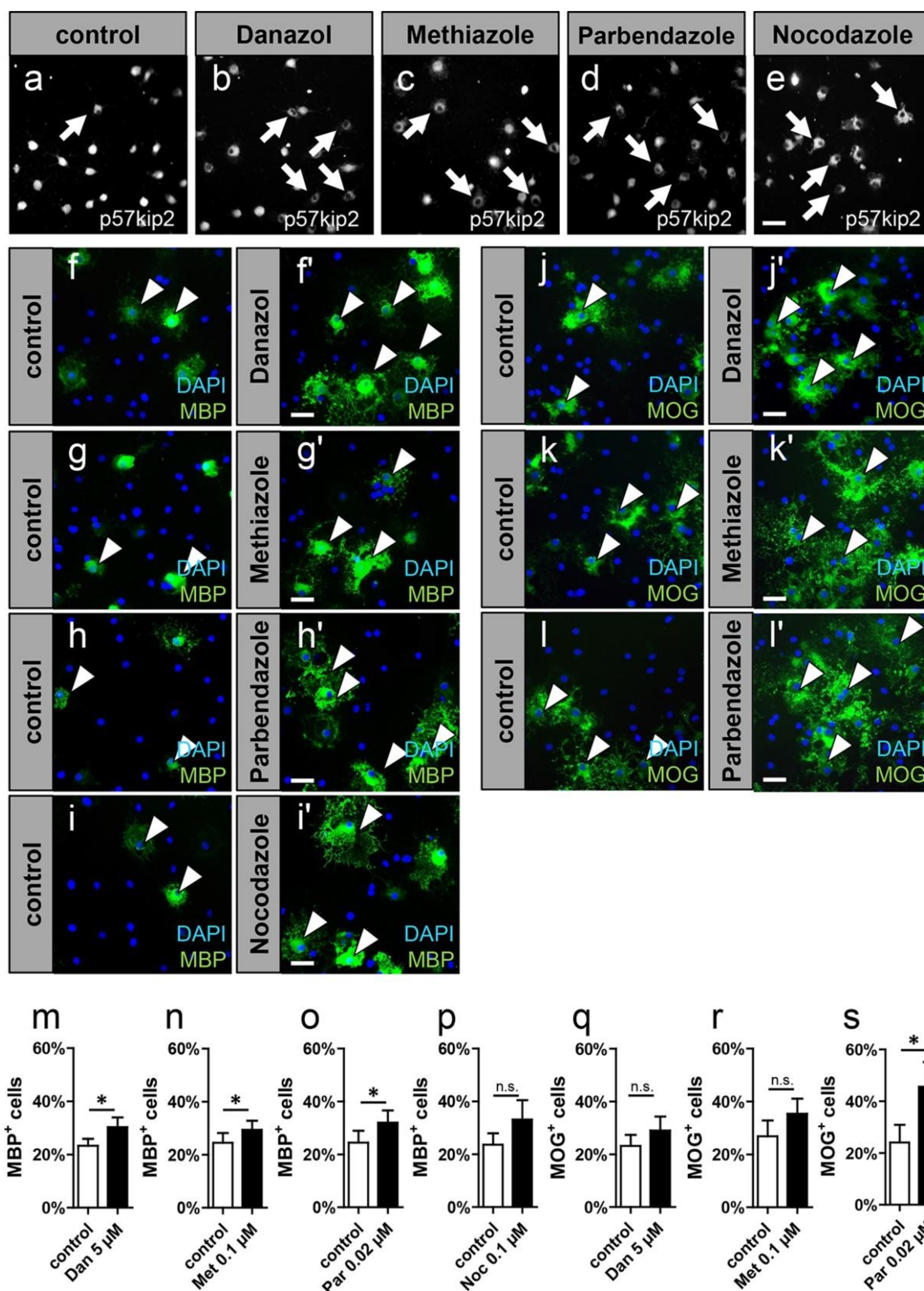


Figure 6. Secondary screening resulted in identification of compounds with long-term OPC differentiation promoting properties. Representative images taken during the primary screening using the BD Pathway 855 High-Content Cell Analyzer visualizing the subcellular localization of the p57kip2 protein upon a 24-hour long stimulation with 0.5% DMSO (a), danazol (b), methiazole (c), parbendazole (d), and nocodazole (e) each at a concentration of 10 µM. Immunocytochemical analysis for the mature myelin marker MBP upon a three-day long stimulation of primary OPCs with 5 µM danazol (m), 0.1 µM methiazole (n), 0.02 µM parbendazole (o), and 0.1 µM nocodazole (p). Immunocytochemical analysis for the mature myelin marker MOG upon a six days long stimulation with 5 µM danazol (q), 0.1 µM methiazole (r), and 0.02 µM parbendazole (s). For each substance as

control are indicated cells stimulated with the corresponding DMSO concentration diluted in differentiation medium. Representative images for MBP (f-i') and MOG staining (j-l'). Arrows in representative pictures point at cells with cytoplasmic p57kip2 localization in a-e and arrowheads point to MBP-positive cells in f-i' or MOG-positive cells in j-l'. Nuclei were counterstained with DAPI. Scale bars: 20 μm. Data are shown as mean values and error bars represent SEM. Numbers of independent experiments n = 6 for (m), n = 4 for (n, p, q), n = 5 for (o, s), n = 3 for (r). Statistical significance was assessed using Mann-Whitney U test, unpaired data: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Dan, danazol; Par, parbendazole; Met, methiazole; Noc, nocodazole; MBP myelin basic protein; MOG myelin oligodendrocyte glycoprotein. Figure 2 from (**Manousi et al., 2021)** Anastasia, M., Peter, G., Laura, R., Qiao-Ling, C., Luke, M. H., Rainer, A., Joel, G., Jessica, S.-H., Jack, P. A., Hans-Peter, H., & Patrick, K. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/https://doi.org/10.1016/j.ebiom.2021.103276.

Following are presented preliminary results of the validation of the rest of primary hits in terms of MBP expression upon a three-day long treatment (Fig. 7). Of note, the here presented graphs depict the most promising, in terms of viability and MBP-induction, results that emerged upon testing of the corresponding compounds at concentrations of 10, 5, 1, and 0.1 μM. The complete data set emerged from this secondary screening is presented in Annex; paragraph 8.4.2. More thorough investigation including additional replications of independent experiments and concentrations series is required to determine their impact on OPC differentiation. In addition, the compounds hexamethonium dibromide dihydrate, mebeverine hydrochloride, and ifenprodil were not included in this validation and therefore no evidence for their effects can be provided.

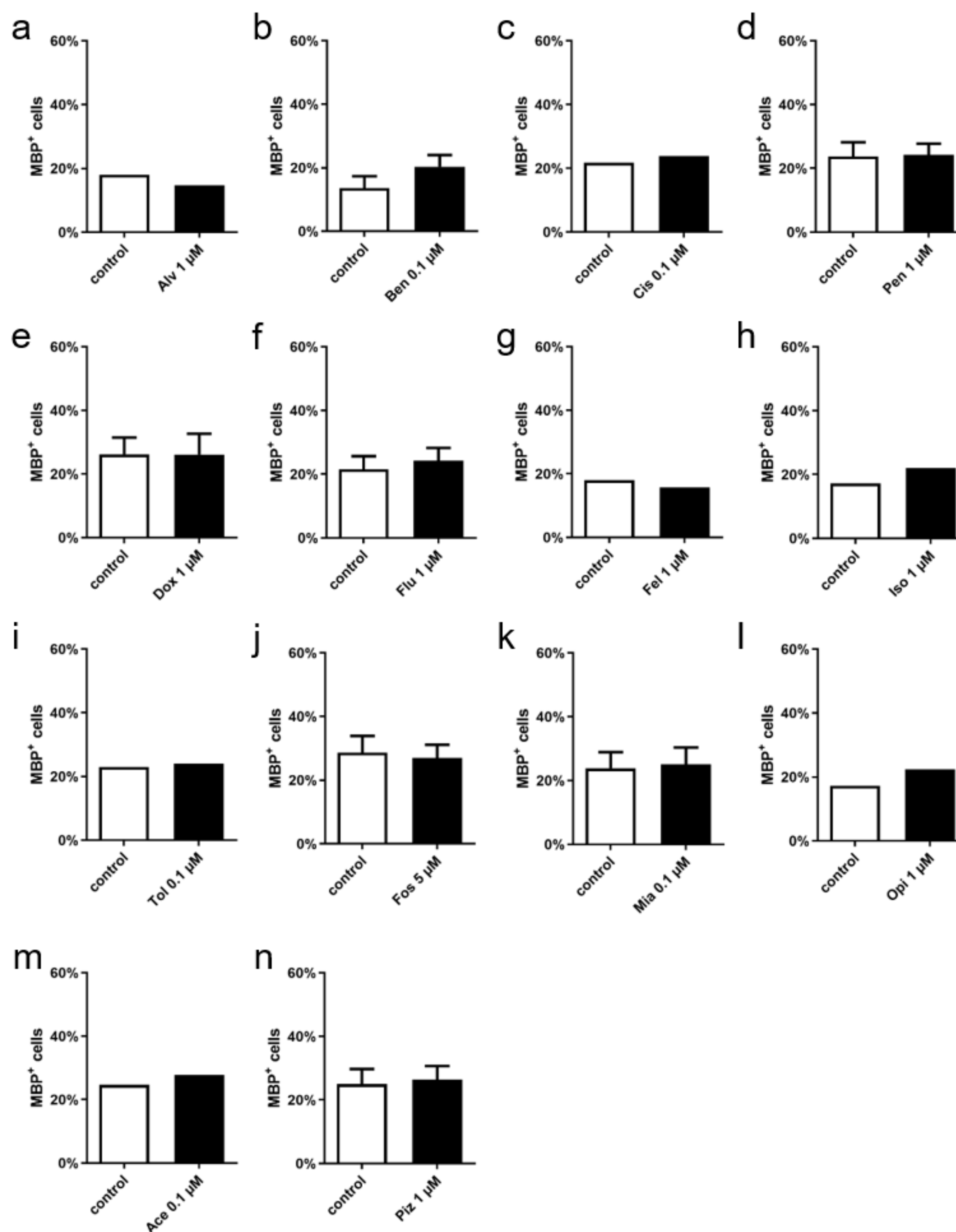


Figure 7. Preliminary results on the capacity of the less promising primary hit compounds to promote OPC differentiation. Immunocytochemical analysis for the mature myelin marker MBP upon a three-day long stimulation of primary OPCs with 1 µM alverine citrate salt (a), 0.1 µM benzamil hydrochloride (b), 0.1 µM cisapride (c), 1 µM pentamidine isethionate (d), 1 µM doxepin hydrochloride (e), 1 µM fluvoxamine maleate (f), 1 µM felodipine (g), 1 µM isoxicam (h), 0.1 µM tolcapone (i), 5 µM fosfosal (j), 0.1 µM mianserine hydrochloride (k), 1 µM opipramol dihydrochloride (l), 0.1 µM acetopromazine maleate salt (m), 1 µM pizotifen malate (n). Data are shown as mean values and error bars represent SEM. Numbers of independent experiments n = 1 for (a, c, g, h, i, l, m), n=2 for (j, k), n=3 for (d, e, f, n), n=4 for (b).

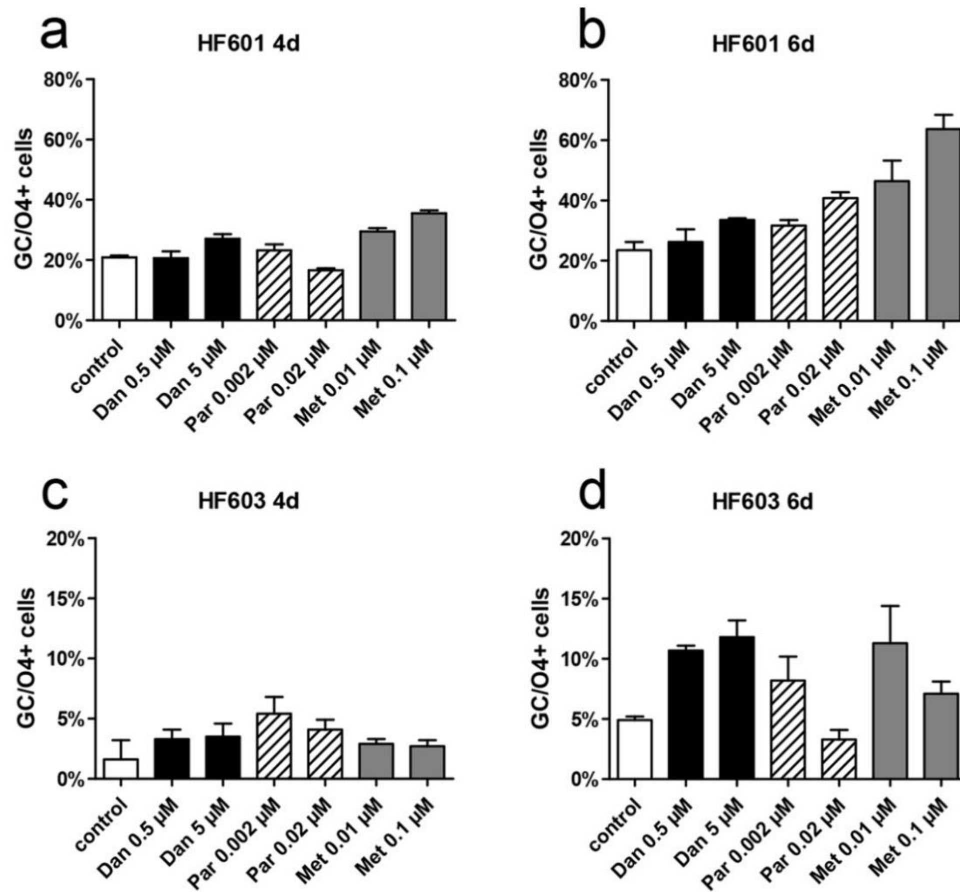
4.3. Selected hit compounds promote oligodendroglial cell differentiation in primary human fetal OPCs[3]

To determine the translational potential of the identified promyelinating compounds, their effects on human cells were investigated. These experiments were conducted by our collaborators Prof. Antel, Dr. Cui and Dr. Healy (Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC H4A 3K9, Canada). To do so, human fetal brain-derived O4-selected oligodendroglial progenitor cells (Cui et al., 2012; Cui et al., 2010; Leong et al., 2014) were grown for four days and then stimulated for another four or six days with parbendazole (0.002 µM and 0.02 µM), methiazole (0.01 µM and 0.1 µM), or danazol (0.5 µM and 5 µM) (Fig. 8a-d). Nocodazole was excluded from this analysis due to the detected cytotoxic effects in rat OPC culture. For technical reasons in total two completely different preparations from human donor tissues were used (HF601 and HF603) which resulted in quantitatively diverging expression levels. For this reason, calculation of mean values and statistical analysis were avoided to prevent false estimations of population variances and low statistical power.

However, double staining with anti-O4 and anti-galactocerebroside (GC) antibodies revealed that all three substances clearly induced an increase in the percentage of morphologically matured human oligodendroglial cells (compare Fig. 8e'',8f'',8g'',8h'') as well as their ability to express GC. Cells of batch HF601 consistently showed increased degrees of anti-galactocerebrosidase (GC)-positivity at time points four and six and using all tested substance concentrations (Fig. 8a,b). Human OPCs of batch HF603 which were generally less mature, showed increased GC-positivity after four days of treatment but appeared sensitive to high parbendazole and methiazole concentrations (Fig. 8c,d). In summary, the most consistent effects were observed for danazol 5 µM both at time points four and six days, whereas the higher concentrations of parbendazole and methiazole resulted in a reduction of the cell numbers of the second preparation (HF603), particularly at the later time point. This suggests additional titration and long-term toxicology investigations to be

[3] These results are published in Manousi et al, 2021; see chapter 8.1

conducted in the future to further assess the extent of these effects. Nevertheless, these data suggest time- and dose-dependent effects of hit compounds with only high danazol concentrations inducing progressively increasing enhance of cell differentiation and parbendazole and methiazole exerting a strong positive regulatory role at low concentrations, which leads to an overall net acceleration of lineage progression.


a
HF601 4d
b
HF601 6d
c
HF603 4d
d
HF603 6d
GC/O4+ cells
control
Dan 0.5 µM
Dan 5 µM
Par 0.002 µM
Par 0.02 µM
Met 0.01 µM
Met 0.1 µM

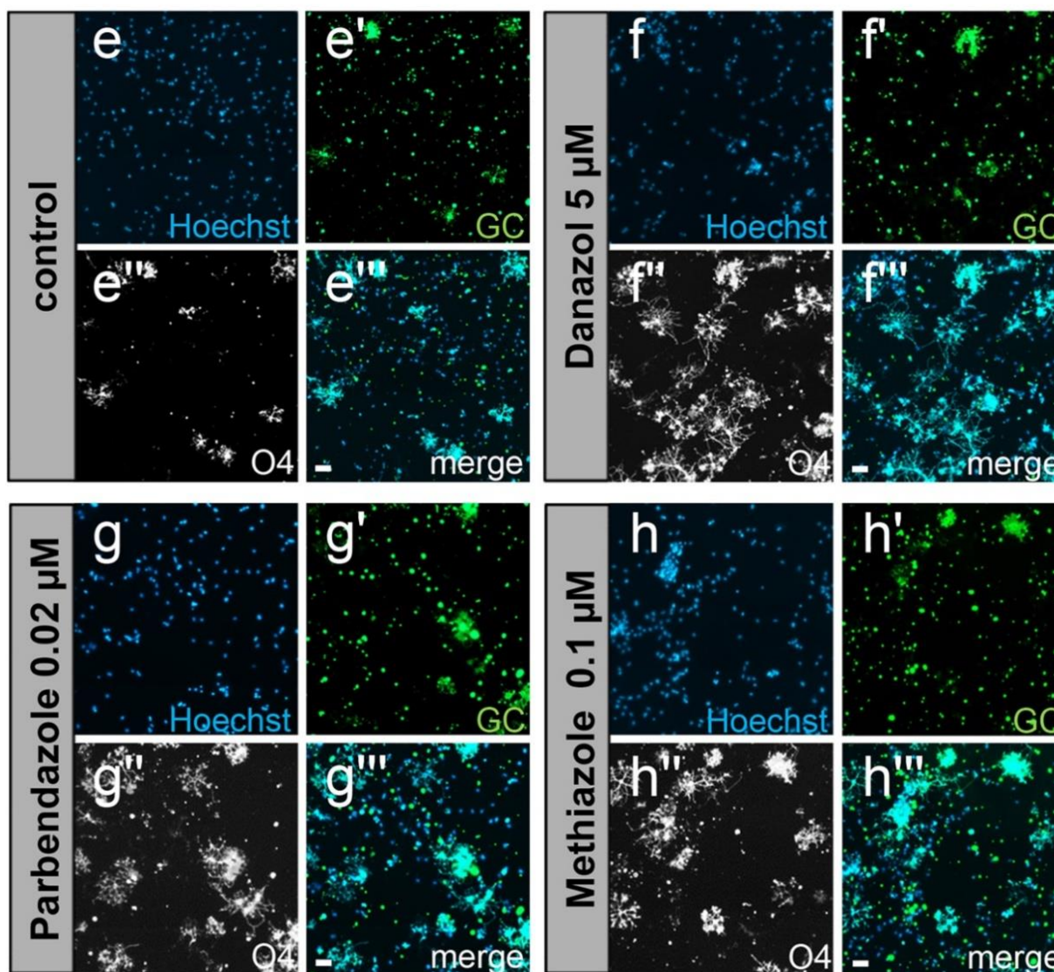
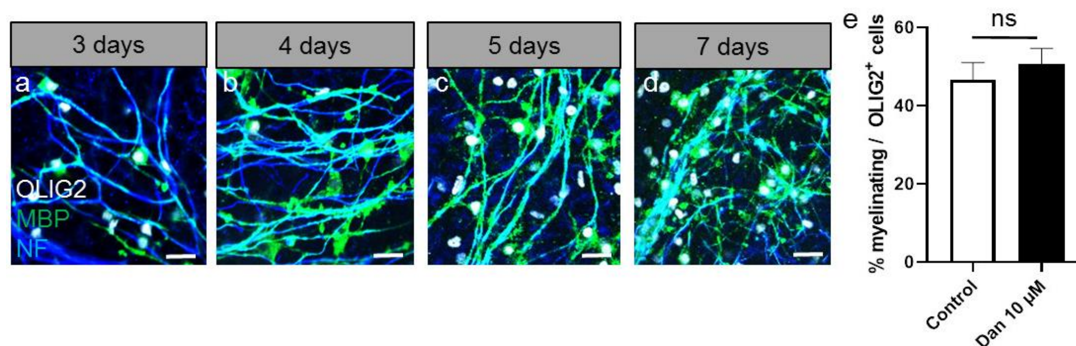

control
Danazol 5 µM
Parbendazole 0.02 µM
Methiazole 0.1 µM
Hoechst
GC
O4
merge

Figure 8. Selected hit compounds promote primary human fetal OPC differentiation *in vitro*. Human OPCs of two different preparations (HF601, HF603) were cultured in the presence of 0.5 μM and 5 μM danazol, 0.002 μM and 0.02 μM parbendazole, or 0.01 μM and 0.1 μM methiazole, or DMSO as control. The results of each preparation are presented separately (HF601 in a,b and HF603 in c,d) and correspond in total to two independent experiments. Representative pictures of HF601 cells after six days of treatment (e-f'''). Note that all three substances induced a clear morphological maturation of cells. Data are shown as means of a single experiment (derived from three replicates per condition) but due to large variations and the still rather low number of repetitions no statistical significance was calculated. Scale bars 20 μm. Figure 3 from (**Manousi et al., 2021**) Anastasia, M., Peter, G., Laura, R., Qiao-Ling, C., Luke, M. H., Rainer, A., Joel, G., Jessica, S.-H., Jack, P. A., Hans-Peter, H., & Patrick, K. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/https://doi.org/10.1016/j.ebiom.2021.103276.

4.4. Members of the benzimidazole class of compounds accelerate developmental myelination *ex vivo*[4]

As parbendazole, methiazole and danazol exerted a positive impact on *in vitro* oligodendroglial cell differentiation both in the rat and human system it was of interest to investigate whether they can also exert similar effects on developmental myelination. To do so again the rat system was chosen and specifically, *ex vivo* organotypic cerebellar slices cultures from Wistar rats aged seven days. The number of OLIG2/MBP-positive myelinating oligodendrocytes within the slices was assessed according to specific morphological criteria (Fig. 10a-a'''). To find the appropriate timepoint of investigation slices were prepared, cultured, and fixated after three, four, five, and seven days in culture medium. Viability did not seem to be affected within a week, however, myelination appeared to be saturated at a great extent from day four on (Fig. 9a-d).



[4] Parts of these results are published in Manousi et al, 2021; see chapter 8.1

Figure 9. *Ex vivo* cerebellar slice cultures are being rapidly myelinated within one week in culture. Organotypic cerebellar slices from P7 rats were cultured in medium for three, four, five, and seven days. Representative triple staining for OLIG2, MBP, and neurofilament (a-d). Scale bars: 20 µm. Immunohistochemical analysis assessing the number of OLIG2/MBP-positive, myelinating oligodendrocytes in relation to the total number of OLIG2-positive cells in slices treated with 10 µM danazol for four days and the corresponding DMSO concentration (control) (e). Data are shown as means and error bars represent SEM. Mann-Whitney U test, for unpaired data was used and data would be considered statistically significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Number of experiments: n = 6. Dan, danazol; NF, neurofilament; MBP, myelin basic protein.

Therefore, the effects of different applied concentrations of parbendazole (0.1 µM), methiazole (1 µM and 2 µM) and danazol (5 µM and 10 µM) were investigated after 3 days of stimulation. Application of either 0.1 µM parbendazole or 2 µM methiazole induced a significant boost of myelination conducted by the endogenous oligodendroglial cell population as assessed by means of the capacity of OLIG2-positive cells to make up MBP-positive myelin sheaths and compared to the control (DMSO) conditions (Fig. 10b-g). The results from the application of 1 µM methiazole were insignificant and they are presented in Annex; paragraph 8.4.3. Application of 10 µM danazol revealed to exert the most prominent effects when compared to the other applied concentration (see Annex; paragraph 8.4.3) and a similar trend regarding the generation of myelinated segments to the parbendazole and methiazole. Nevertheless, no significant difference was detected (Fig. 10h). When the same concentration of danazol was then applied for 4 days, the effect on myelination was even less prominent, thus supporting the initial observation that myelination reaches already within 4 days a maturation level which does not favor the detection of effects on the process upon pharmacological modulation (Fig 9e).

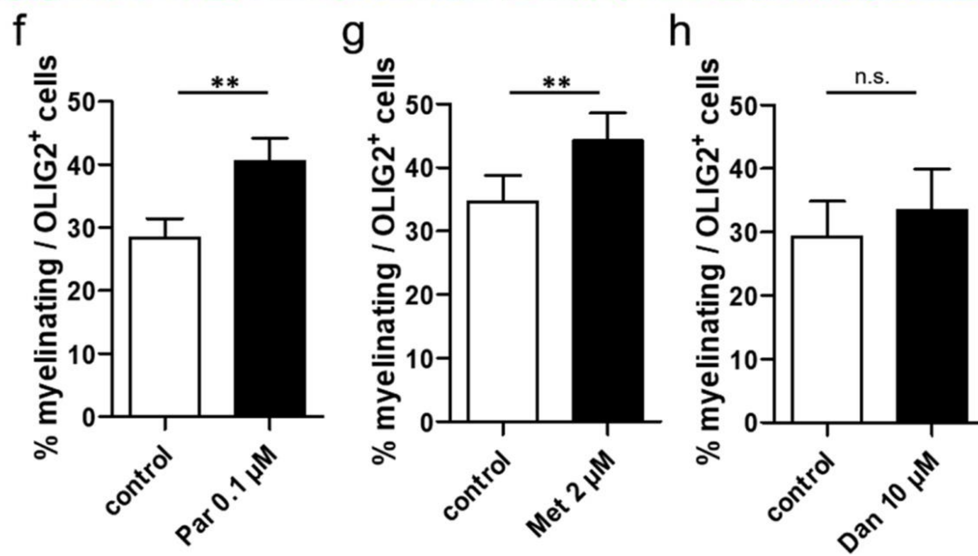

MBP positive myelinating cell
a
a'
a''
a'''
merge
MBP
NF
OLIG2
control
b
OLIG2
MBP
NF
c
c'
c''
c'''
OLIG2
NF
MBP
merge
Parbendazole 0.1 µM
d
OLIG2
MBP
NF
e
e'
e''
e'''
OLIG2
NF
MBP
merge
f
g
h
% myelinating / OLIG2+ cells
**
**
n.s.
0
10
20
30
40
50
control
Par 0.1 µM
Met 2 µM
Dan 10 µM

Figure 10. Parbendazole and methiazole promote *ex vivo* developmental myelination. Organotypic cerebellar slices from P7 rats were stimulated with the hit compounds for three days. Exemplary pictures of a myelinating oligodendrocyte showing the morphological criteria used for the analysis (a-a’’’). Representative triple staining for OLIG2, MBP, and neurofilament for DMSO (b-c’’’) and parbendazole- (d-e''') treated slices. Scale bars for the exemplary images (a-a’’’): 10 µm. Scale bars for the overview images (b,d): 100 µm. Scale bars for the detailed images (c-c'''; e-e'''): 20 µm. Immunohistochemical analysis assessing the number of OLIG2/MBP-positive, myelinating oligodendrocytes in relation to the total number of OLIG2-positive cells in slices treated with parbendazole (f), methiazole (g), danazol (h), and their corresponding DMSO concentrations (controls). Data are shown as means and error bars represent SEM. Mann-Whitney U test, for unpaired data was used and data were considered statistically significant at *p < 0.05, **p <0 .01, ***p < 0.001. Number of experiments: n = 5 in all treatments. Par, parbendazole; Met, methiazole; Dan, danazol; NF, neurofilament; MBP, myelin basic protein. Figure 4 from (**Manousi et al., 2021**) Anastasia, M., Peter, G., Laura, R., Qiao-Ling, C., Luke, M. H., Rainer, A., Joel, G., Jessica, S.-H., Jack, P. A., Hans-Peter, H., & Patrick, K. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/https://doi.org/10.1016/j.ebiom.2021.103276.

4.5. Parbendazole and danazol enhance remyelination *in vivo*[5]

To determine whether the hit compounds parbendazole and danazol have the potential to promote myelin repair, their impact on spontaneous remyelination was assessed. To this goal, the cuprizone-mediated mouse model of de- and remyelination was applied. This is a well-established method for identification of compounds able to boost the endogenous remyelination capacity of the CNS (Matsushima & Morell, 2001; Zhan et al., 2020). Given that *in vivo* experiments are time consuming and require a significant number of experimental animals, out of the three prominent hit compounds (danazol, parbendazole and methiazole) which emerged from the previous validation experiments we excluded methiazole from the first phase of these experiments since it belongs to the same chemical family as parbendazole, which showed the overall stronger potential both *in vitro* and *ex vivo*. Demyelination was achieved via 0.2% cuprizone diet for six weeks. Daily compound administration was performed during the last 17 days of cuprizone treatment. Parbendazole (20mg/kg) was applied intraperitoneally (i.p.) and danazol (100mg/kg) orally in drinking water. For each compound, the same number of cuprizone-treated mice were administered

[5] These results are published in Manousi et al, 2021; see chapter 8.1

the corresponding vehicles. Body weight was in general increasing during the first four weeks and was mostly stable thereafter, indicating good tolerability of all treatments. No obvious behavioral changes were observed. To assess both the efficiency of cuprizone-mediated demyelination and the extent of remyelination, naïve (non-cuprizone-treated) animals of the same age were also included in the analysis. The effect of the compounds on remyelination was investigated one week upon cuprizone withdrawal (Fig. 11a).

First the efficiency of the cuprizone treatment was confirmed by the detection of significant differences in the percentage of PLP-positive (myelinated) area along the midline of the corpus callosum, following comparison of naïve with vehicle-treated groups (Fig. 11b,b’,f,g,g’,h). The same readout showed significant acceleration of spontaneous remyelination upon parbendazole administration in comparison to vehicle-treated counterparts (Fig. 11b-b'',f). Moreover, a positive effect of the treatment on oligodendroglial cell development was revealed by a significant increase in the number of cells expressing the mature oligodendrocyte protein markers GSTπ (Fig. 11c-c'',f') and CC1 (Fig. 11d-d'',f'') within the region of interest. This observation was supported by the opposite expression pattern of the early oligodendroglial marker PDGFRα, where the vehicle-treated group demonstrated significantly increased numbers of PDGFRα-positive cells as compared to the other two groups (naïve and parbendazole-treated mice, Fig. 11e-e'',f'''). No significant changes in the numbers of OLIG2-positive cells and almost no proliferative cells (Ki67-positive) were detected in the region of interest (data not shown). Interestingly, after one week of remyelination the PLP-positive area along the midline of the corpus callosum in danazol-treated animals was also significantly enhanced in comparison to the vehicle-treated ones (Fig. 11g-g'',h). This positive effect on remyelination was accompanied by a statistically significant increase in the generation of GSTπ-positive cells, whereas numbers of CC1- and PDGFRα-positive cells remained at the same levels (Fig. 11h'-h''').

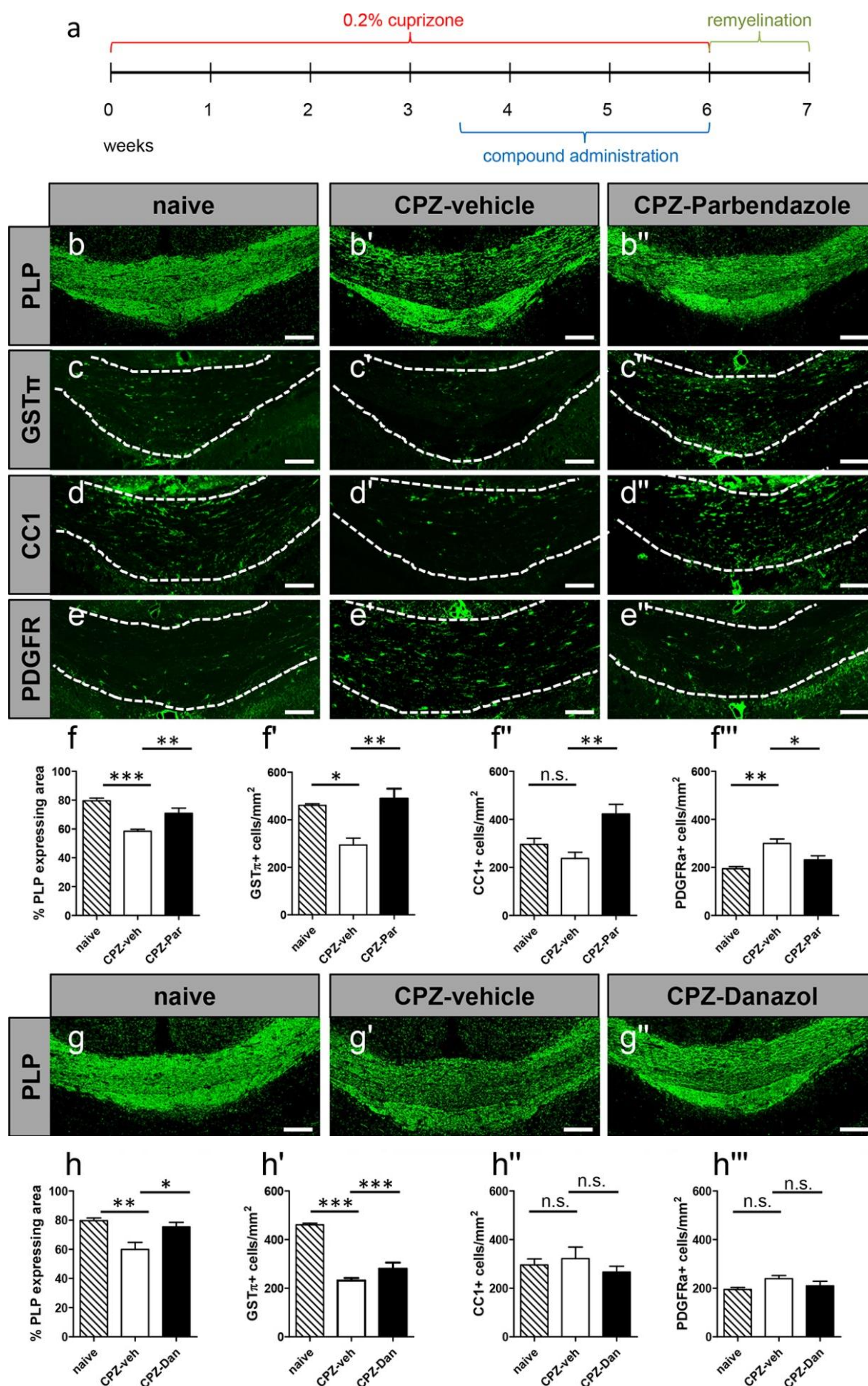


Figure 11. Parbendazole and danazol promote spontaneous remyelination in a cuprizone-induced demyelination mouse model. Representation of the application scheme both for cuprizone treatment and compound administration (a). Representative images of the oligodendroglial protein marker expression along the midline of corpus callosum upon 1 week of remyelination (b-e''; g-g'').

Image magnification: 10x. The extent of remyelination was revealed as differences in the percentage of PLP-expressing (myelinated) area in the defined region of interest among naïve, CPZ/vehicle-treated and CPZ/substance-treated groups. Similarly, the impact of parbendazole and danazol on oligodendroglial cell differentiation and maturation was assessed by the number/mm^2 of mature (GSTπ, f’,h’; CC1, f'',h'') and early (PGFRα, f''',h''') oligodendroglial protein marker-positive cells along the region. Naïve group n = 6 (n = 4 for the GSTπ analysis); residual groups n = 5 each. For each mouse 4 coronal sections were analyzed (2 from the medial and 2 from the caudal compartment). Data are shown as mean values and error bars represent SEM. Significance (95% confidence interval) was assessed using Tukey's range test following one-way ANOVA. Data were considered statistically significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. CPZ, cuprizone; veh, vehicle; Par, parbendazole; Dan, danazol; PLP, myelin proteolipid protein; GSTπ, glutathione-S-transferase Pi; CC1, adenomatous polyposis coli protein (APC) clone CC1; PDGFRα, platelet-derived growth factor receptor alpha. Scale bars: 100 µm. Figure 5 from (**Manousi et al., 2021**) Anastasia, M., Peter, G., Laura, R., Qiao-Ling, C., Luke, M. H., Rainer, A., Joel, G., Jessica, S.-H., Jack, P. A., Hans-Peter, H., & Patrick, K. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/https://doi.org/10.1016/j.ebiom.2021.103276.

4.6. Parbendazole treatment induces early activation of p38MAPK *in vitro*[6]

After clearly showing an early positive effect of parbendazole on oligodendroglial lineage progression both in development and in myelin repair, available information about benzimidazole derivatives and parbendazole, in particular, were used to investigate the underlying mechanism of action in these experiments. Interestingly, p38MAPK activation has been associated with regulation of nuclear protein export (del Arco et al., 2000; Gomez et al., 2011; Seternes et al., 2002). Moreover, another benzimidazole derivative, namely omeprazole, was shown to induce *in vitro* OPC differentiation involving p38MAPK and ERK1/2 activation during early stages of the process (Zhu et al., 2019). In order to investigate whether p38MAPK is also activated in response to parbendazole in our primary culture, OPCs were stimulated with this substance for 60 minutes and indeed 2.3 times more phosphorylated p38MAPK protein in relation to the DMSO-treated control cells was detected (Fig. 12).

[6] These results are published in Manousi et al, 2021; see chapter 8.1

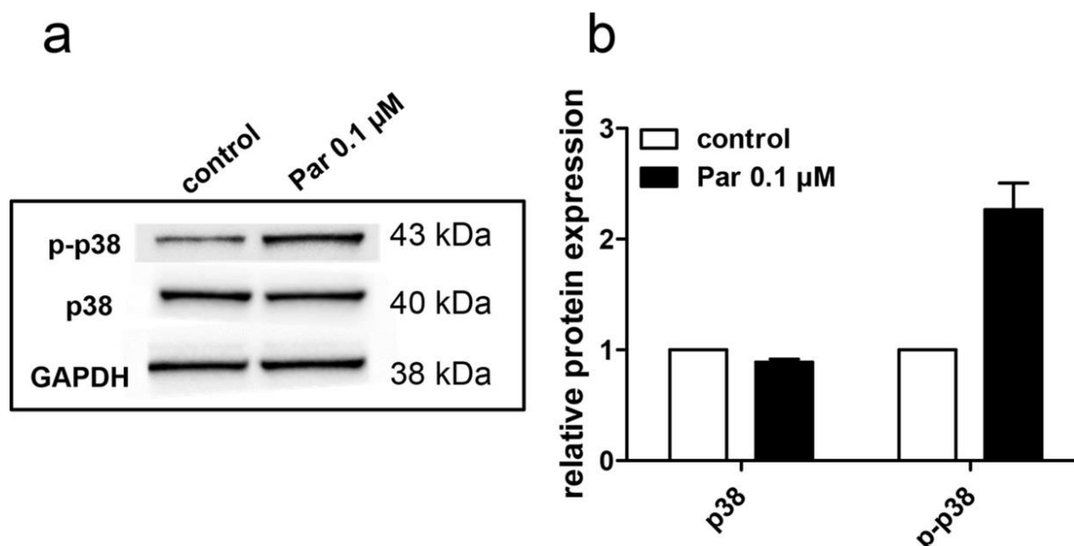


Figure 12. OPC stimulation with parbendazole induces phosphorylation of p38MAPK. Rat primary OPCs were treated either with 0.01 μM parbendazole or the corresponding DMSO concentration (control) in differentiation medium for 60 minutes and harvested for Western blot analysis for the detection of p-p38, p38, and GAPDH proteins (a). Quantification of the relative protein expression (b). The relative protein expression of both the phosphorylated and non-phosphorylated forms of p38 was normalized over the total amount of the protein in the lysate and GAPDH expression. Data represent means of two independent experiments, while error bars represent SEM. Par, parbendazole; kDa, kilodalton. Figure 6 from (**Manousi et al., 2021**) Anastasia, M., Peter, G., Laura, R., Qiao-Ling, C., Luke, M. H., Rainer, A., Joel, G., Jessica, S.-H., Jack, P. A., Hans-Peter, H., & Patrick, K. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/https://doi.org/10.1016/j.ebiom.2021.103276.

5. DISCUSSION

Multiple sclerosis is the most common demyelinating disease in which an altered immune response leads to oligodendrocyte death and myelin destruction, thus resulting to neurodegeneration and permanent neurological damage (Noseworthy, 1999; Trapp & Nave, 2008). While the currently used treatments mainly act immunomodulatory and slow down the disease progression, lately the scientific interest is shifted towards the development of pharmacological approaches which can support the innate capacity of the CNS to repair the already existed damage (Kremer et al., 2019b; Kremer et al., 2019c). Specifically, a resident population of OPCs in the adult CNS is capable of differentiation and remyelination of demyelinated neurons, events that lead to neuroprotection, rapid signal transduction, and inversion of existed neuronal deficits (Dawson et al., 2003; Franklin & ffrench-Constant, 2017). However, the innate remyelination capacity of the CNS is inefficient and declines with disease progression, mainly due to inefficient OPC activation and differentiation to myelinating oligodendrocytes (Gruchot et al., 2019; Kuhlmann et al., 2008). Therefore, identification of compounds that promote OPC differentiation appears to be an effective approach for the treatment of demyelinating diseases such as MS. Although many substances have been repurposed towards this goal, with some of them, such as clemastine, to have already entered clinical trials (Kremer et al., 2019c), an efficient treatment for myelin repair is yet an unmet goal.

In this respect, this doctoral thesis aimed at the identification of myelin repair promoting compounds. To this end, a large-scale screening based on the known inhibitory role of the p57kip2 protein in oligodendroglial cell differentiation (Göttle et al., 2015) was established and conducted. Specifically, 1280 known small molecules were tested for their ability to promote nuclear exclusion of the p57kip2 protein, a process which is associated with OPC differentiation and maturation. From this primary screening 21 compounds with these properties were identified and then validated in terms of their potential to promote cell differentiation in rat primary OPC cultures. Four compounds were found to significantly accelerate transition of OPCs to mature oligodendrocytes *in vitro* and for three of them well-

tolerated concentrations were defined. These compounds, namely parbendazole, methiazole and danazol positively affected differentiation of human fetal OPCs and developmental myelination in rat cerebellar organotypic brain slices. Using a mouse cuprizone-induced de- and remyelination model the myelin repair properties of parbendazole and danazol were investigated. Administration of both substances resulted in significant acceleration of remyelination and OPC differentiation in corpus callosum of intoxicated mice. As proof of concept, it was shown that the early regulatory effect of parbendazole treatment *in vitro* was associated with activation of the p38MAPK protein (Manousi et al., 2021).

5.1. Large scale screening for pharmacological compounds with the potential to promote p57kip2's nuclear exclusion and OPC differentiation

A significant number of unbiased large scale screening approaches, like the one described in this thesis, use large numbers of available active compounds and the existing knowledge about them, to identify and repurpose a few of them for myelin repair. These studies are either experimental or in silico. The experimental ones can be whole organism-based using testing on zebrafish (Buckley et al., 2010; Early et al., 2018; Preston et al., 2019) or cell-based using either OPC cultures (Deshmukh et al., 2013) or myelinating systems (Bijland et al., 2019; García-León et al., 2018; Lariosa-Willingham et al., 2016b; Mei et al., 2014) and they have been proven to be effective for the identification of compounds promoting OPC differentiation and survival or even directly myelination. In silico virtual screenings have also been applied for the identification of compounds with promyelinating properties (Parravicini et al., 2020).

Given that the rate limiting factor which leads to inefficient remyelination in demyelinating diseases is the limited capacity of adult OPCs to differentiate into myelinating oligodendrocytes, cell-based large-scale screenings which aim to identify compounds that promote this process constitute a powerful approach for the development of myelin repair therapies. In addition, the already existed information about these compounds can not only accelerate the process of drug development but it can also be used to shed light to the

underlying regulatory mechanisms of the transition from OPCs to mature oligodendrocytes. Taking this into account a collection of mainly FDA/EMA- (US Food and Drug Administration/Europeans Medicines Agency) approved compounds was chosen for this thesis. In addition, 98% of these small molecules were predicted to have a high blood-brain-barrier (BBB) penetrance, a characteristic very crucial for the translational significance of this study given that the cell targets of such a therapy are exclusively localized in the CNS. Of note, the tested compounds in this study constitute an enriched version of the Prestwick Library used in two other screening setups for identification of OPC differentiation promoting drugs, however using different cell types and readouts (Buckley et al., 2010; Porcu et al., 2015). The 23 primary hits which emerged from the screening established by Porcu et al. were directly assigned to different categories according to their biological relevance and the mechanistic insights of the mode of action of two prominent ones, halcinonide and clobetasol, were described. Moreover, the three dose-response strategy (1 μM, 10 μM, and 25 μM) of this screening provided information regarding the dose-dependent tolerability and effectivity of the compounds in respect to OPC differentiation. This prior knowledge contributed to the decision to catholically apply the concentration of 10 μM in the here described screening, a restriction that had to be made for practical reasons. Similarly, the use of the same library in a screening that involved direct compound application to larval zebrafish again led to identification of 25 compounds with promyelinating properties which were assigned to different categories according to their known cellular and molecular properties (Buckley et al., 2010), thus indicating the potential of this compound collection in the field of myelin repair.

Although the choice of this compound library led to successful screenings in both two previously discussed two studies and in the screening described in this thesis, high-throughput or targeted screenings may require more sophisticated methods for the library selection to increase their efficiency. This goal can be achieved via determination of the structural and topological uniqueness of each compound to avoid redundancy using cheminformatics tools like the Bemis-Murcko scaffold analysis (Bemis & Murcko, 1996) and

comparison of the characteristics of the commercially available libraries and compounds in databases for virtual screenings and catalogs of vendors, as for example the ZINC database (Sterling & Irwin, 2015).

Most of the published cell-based screenings for pharmacological compounds promoting oligodendrogenesis used either stem cell-derived OPCs (Allimuthu et al., 2019; Cui et al., 2019; Guo et al., 2018; Hubler et al., 2018; Najm et al., 2015; Suo et al., 2019) or cell lines (Joubert et al., 2010; Peppard et al., 2015; Porcu et al., 2015) and only a few used primary rodent OPC cultures (Chen et al., 2017; Deshmukh et al., 2013; Eleuteri et al., 2017; Lariosa-Willingham et al., 2016a; Rosler et al., 2016). The here presented screening is based on compound testing on primary cells, a condition closer related to the *in vivo* situation, as compared to cell lines and stem cell-derived cultures, to increase the translational significance of the findings.

A common feature of most of these screening approaches is the use of MBP both on gene and protein level as readout (Allimuthu et al., 2019; Chen et al., 2017; Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Hubler et al., 2018; Lariosa-Willingham et al., 2016a; Mei et al., 2014; Najm et al., 2015; Porcu et al., 2015; Suo et al., 2019). Some others used morphological maturation (Joubert et al., 2010; Peppard et al., 2015) and changes in metabolic activity (Eleuteri et al., 2017) as readouts instead. In all cases a long-term stimulation of at least two days was used and they aimed at identification of compounds that promote transition from the OPC stage to mature myelinating oligodendrocytes. This study on the contrary aimed to identify effects resulted only after 24 hours upon the initiation of the compound stimulation. The use of this novel readout, p57kip2´s protein subcellular localization, allowed investigation of regulatory events happening early during differentiation, within a time-window much smaller than the ones used by other screenings.

The effectivity of this approach was proven by the fact that some of the identified primary hits are known to modulate molecular mechanisms which are implicated in the regulation of OPC differentiation (Cheli et al., 2015; Chen et al., 2014; Fan et al., 2015; Preisner et al., 2015).

These compounds are either nonsteroidal anti-inflammatory drugs (NSAIDs; e.g. fosfosal) or ion channel modulators (e.g. benzamil hydrocloride), and neurotransmitter receptor modulators (e.g. alverine citrate salt). Another proof or concept for this study is the overlap with hits previously detected by other screenings, namely ifenprodil (Hubler et al., 2018; Lariosa-Willingham et al., 2016a; Najm et al., 2015) and isoxicam (Buckley et al., 2010). This overlap, although significant, it was minor which is a fact that can be explained by the use of different cell types, collections of tested compounds and concentrations between other screening assays and this one. Another prominent explanation is the novelty of the readout used in this study which addresses earlier stages of oligodendroglial cell differentiation and possibly also different regulatory mechanisms.

Although the biological significance of the here presented screening approach was validated by the number and the nature of the confirmed hits and the identification of compounds among them with myelin repair properties, the evaluation of the statistical data quality was not satisfactory, as revealed by the negative value of the Z factor, a universally used measure of statistical effect size (Zhang et al., 1999). The major explanation is the use of primary cell culture. Besides the advantages of such a culture on a translational level, it is characterized by the drawback of heterogeneity among independent experiments. Differences in the maturation stage of the isolated OPCs and their differentiation potential, in combination with the narrow window of time that p57kip2`s protein nuclear exclusion takes place, may have led to an overlook of compound- and control-induced effects. In addition, even though all the parameters that could affect cell behavior were kept stable throughout the whole screening procedure, it should be noted that all the experimental procedures were performed in a non-automated way. Another reason that may have caused missed effects or failure to reproduce the already detected ones is the single applied concentration of each tested compound. Approaches to overcome this limitation used by others, constitute the multiple-dose strategy (Porcu et al., 2015) and performance of toxicity assays prior to the primary screening (Lariosa-Willingham et al., 2016a).

Four out of 21 confirmed primary hits significantly increased transition from the OPC stage to mature oligodendrocytes and these effects were detectable throughout the whole process. Nuclear localization of the p57kip2 protein is associated with early stages of oligodendroglial cell differentiation, while cytoplasmic p57kip2 is detected in more mature, differentiation competent cells (Kremer et al., 2009). Moreover, enforced nuclear exclusion or overexpression of the protein affect the lineage progression (Göttle et al., 2015). Taken these findings into account more hits could be expected to be characterized to induce a greater differentiation potential. An explanation for the small number of hit compounds that promoted oligodendrogenesis in this study (see results section; paragraph 4.2 and Annex; paragraph 8.4.2) is that for this secondary screening only MBP protein expression at day three was investigated and therefore, critical time points of the effect for some compounds may have been missed. In addition, inappropriate tested concentrations, or activation at later stages (later than the 24 hours of stimulation during the screening procedure) of additional adverse pathways which interfered with cell differentiation or viability may also explain the absence of the desired effects. More thorough validation of the primary hits is needed to further assess their impact in lineage progression. An effective and time saving way to do so is to first calculate the EC50 value via generation of a dose-response curve and fitting the data to a dose-response function using the appropriate software. This is an approach which has been used in several screening approaches to rank primary hits according to their potency and then prioritize them accordingly for further validation (Chen et al., 2017; Deshmukh et al., 2013; Lariosa-Willingham et al., 2016a, 2016b; Najm et al., 2015; Parravicini et al., 2020; Peppard et al., 2015; Porcu et al., 2015; Rosler et al., 2016).

Despite the limitations of the method, this screening not only led to the identification of compounds with promyelinating properties but also clearly suggested a novel screening readout which can further lead to drug repurposing for myelin repair when applied in large scale screenings with modified setups. This notion is also supported by a recent publication from our research group, which suggests that teriflunomide not only exerts

immunomodulatory effects but also promotes p57kip2 shuttling along with concomitant OPC differentiation and myelination *in vitro* (Göttle et al., 2018).

5.2. Translation to the human system

Translation of the findings from other systems into the human is a crucial determinant for a successful screening for therapeutic compounds with pro-myelinating properties. OPC differentiation in the rodent and the human system share many similar regulatory mechanisms and surface marker expression profiles. However, there are significant differences in their transcriptome, as well as in the temporal aspects and regulation both of oligodendroglial cell development and myelination (Pol et al., 2017), reviewed in (Barateiro & Fernandes, 2014; Dietz et al., 2016). Hence, including cells of human origin in such a study is essential for the determination of treatment-induced effects on species-conserved regulatory signaling pathways. As previously discussed, most of the published screenings in this field are based on the use of rodent cells and only a few of them have addressed this aspect. Specifically, human OPCs generated from skin fibroblast-derived human iPSC line and hESC lines (Najm et al., 2015), human glioma cell line (Hubler et al., 2018) and human PMD patient iPSC-derived cells used for generation of oligocortical spheroids (Elitt et al., 2018) have been used to reproduce pharmacological effects on cells of rodent origin.

This study was designed in a way that allows the identification of compounds with modulatory effects that target non-region dependent and evolutionary conserved molecular mechanisms. Precisely, the impact of the confirmed hit application on oligodendroglial lineage development was detected in cells derived from three different brain regions, namely cerebral cortex, cerebellum, and corpus callosum during the progress of this study, thus indicating targeting of globally active regulatory mechanisms. Notably, prior to the initiation of the here presented screening, Göttle et al. using both rat and human primary OPC cultures could show that the regulatory effects of p57kip2 in oligodendroglial cell differentiation consist an evolutionary conserved mechanism (Göttle et al., 2015). Although these findings significantly contributed to the choice of this specific readout for its translational potential, given the multiple possible targets each applied compound may have, it was of essence to

investigate their effects in other systems as well. To this end, the rat, mouse, and human systems were used and the impact of both danazol and the benzimidazole derivatives was to be successfully detected.

The testing on human OPCs was performed in collaboration with Prof. Antel, Dr. Cui and Dr. Healy (Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC H4A 3K9, Canada). Our collaborators tested the impact of danazol, methiazole and parbendazole on human fetal OPCs using a well-established and broadly applied protocol (Cui et al., 2012; Cui et al., 2010; Kremer et al., 2016b; Leong et al., 2014). Fetal OPCs were extracted from telencephalic regions between gestational weeks 14 and 17, which is a timepoint that corresponds to a stage where OPCs can already be observed in the forebrain and they start to increase their numbers in the ganglionic eminences and in the cortical ventricular zone/subventricular zone (Jakovcevski et al., 2009). Of note, this protocol was used in the past in a similar way as in this study, leading to translation of the promoting effects of indomethacin in OPC differentiation from the murine to the human system, thus highlighting its potential as a promising therapy for myelin repair (Preisner et al., 2015). In another study, the use of this cell culture allowed elucidation of the underlying mechanism of fingolimod application in OPC differentiation (Cui et al., 2014). The here presented experiments indeed indicated that the identified hit compounds target evolutionary conserved molecular mechanisms, since all three compounds positively affected differentiation of human fetal OPCs. However, these data emerged from two independent preparations of cells from a different maturation stage, and they were characterized by heterogeneity. Therefore, to specifically describe their impact throughout the process of differentiation more experiments are required. As discussed earlier, as beneficial the use of primary cultures as it is, differences in the maturation stage of the cells or in their differentiation potential, among different experiments it to be expected and for this reason more technical replicates usually are needed to draw a conclusion. In this respect, our collaborators showed in the past that fetal OPCs from different gestational weeks behave differently in cell culture, with oligodendrocyte lineage marker expression and myelination being proportional to age and

the survival rate being inversely proportional (Cui et al., 2012). According to our observations, although both cell preparations were performed around the same gestational week, it cannot be excluded that they represent different developmental stages which are diversely regulated or even distinct populations as previously shown for the rodent system (Kessaris et al., 2006).

5.3. Systematic assessment of hit compound-mediated myelin repair activities

Screening platforms for direct identification of compounds with myelination promoting properties have been established and performed (García-León et al., 2018; Lariosa-Willingham et al., 2016b; Mei et al., 2014) and new technologies are under development (Bijland et al., 2019; Kerman et al., 2015). Myelination is a complex process, dependent on several stages of oligodendroglial lineage development and many regulatory processes, reviewed in (Mitew et al., 2014; Nave & Werner, 2014). Therefore, direct identification of compounds that promote myelination, may not lead directly to information regarding their mode of action and the targeted regulatory processes. In addition, establishing such screenings requires more complex technologies and analytical methods. On the contrary, targeting specifically oligodendroglial cell differentiation in monoculture is easier to be established and ensures higher probability of molecular target and regulatory process determination. Moreover, as inefficient OPC differentiation is the main rate-limiting factor for myelin repair in demyelinating diseases such as MS (Chang et al., 2002; J. K. Huang et al., 2011; Kuhlmann et al., 2008; Wang et al., 2020; Wolswijk, 1998), pharmacological boost of this process holds already a great therapeutic potential in this regard. Such screenings though, should be followed by validation in terms of impact of the hit compounds on myelination to eventually lead to identification of compounds with myelin repair properties. In this respect, co-culture systems (Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Parravicini et al., 2020; Suo et al., 2019), electrospun fibers (Allimuthu et al., 2019; Elitt et al., 2018; Hubler et al., 2018), *in vivo* approaches with neonatal rats (Najm et al., 2015) and organotypic cerebellar slice cultures (Eleuteri et al., 2017; Najm et al., 2015) have led to successful validation of hits with pro-differentiation properties.

Cerebellar slice cultures when compared to *in vitro* cultures, they have the advantage of maintaining their three-dimensional structure, cell-cell and cell-ECM interactions, thus allowing to a great extent replication of the *in vivo* myelination process. The use of cerebellar slices offers temporal and regional defined conditions for reproducible results (Jaeger et al., 1988; Notterpek et al., 1993). In addition, most of the analyzed white tracts come from the middle cerebellar peduncle and they are among the longest in the mammalian brain, an advantageous characteristic for evaluation of myelination (Suzuki et al., 2012). As for the temporal components, in the here presented study the cerebellar slices were prepared at P7, which corresponds to the developmental stage in rodents when white matter tracts contain more than 80% immature oligodendrocytes and the myelination initiates (Dean et al., 2011). While at this stage the first MBP-positive cells start appearing, at day P14 they become abundant, thus suggesting the period around day P7 as the most optimal for testing of compounds with the potential to regulate developmental myelination (Bjelke & Seiger, 1989; Hardy & Friedrich, 1996). Literature suggests, equilibration of the slices for 3 days to allow clearance of debris and stabilization of the slices, and then another 3 or 4 days of compound treatment (Barateiro & Fernandes, 2014). In the experiments of this study, 4 hours of equilibrium were enough for transparency of the slices to be detected and they ensured a better quality of the culture when compared to 24 hours of equilibrium (data not shown). Moreover, to have a defined image of the progress of myelination in our experimental setup, a timeline was conducted (3, 4, 5, and 7 days) and showed that even after 4 days *in vitro,* myelination was in an advanced level. Such differences in the lineage progression and myelination may emerge from minor adaptations in the protocol, as for example thickness of the slices or glucose concentration in the medium, or due to different handling of the slices during preparation. Therefore, each experimenter should keep these parameters constant and thoroughly investigate the behavior of the slices in his hand before initiation of an experimental procedure.

Although developmental myelination and remyelination after injury share many common aspects, the latter cannot be considered recapitulation of the first (Franklin & ffrench-

Constant, 2008; Taveggia et al., 2010). Upon comparison between fetal and adult human OPCs was shown that they are characterized by differential miRNA expression and that the later are even comprised of phenotypically distinct subsets, therefore suggesting different regulatory mechanisms of OPC differentiation in the adult brain (Leong et al., 2014). On that account, investigation of the applied compounds on developmental myelination is not sufficient for determination of their translational potential as therapeutics for demyelinating disorders. To this goal, a few different animal models have been developed for the investigation of OPC development in injury and disease. Specifically, as previously introduced, evaluation of the impact of hit compounds on remyelination is most of the times the final validation step upon screenings for compounds with myelin repair properties (Chen et al., 2017; Cui et al., 2019; Deshmukh et al., 2013; Eleuteri et al., 2017; Guo et al., 2018; Hubler et al., 2018; Mei et al., 2014; Najm et al., 2015; Parravicini et al., 2020; Suo et al., 2019; Zhu et al., 2019). For this purpose, in this study the well-established cuprizone-mediated demyelination model was chosen, based on the little or no implication of adaptive immunity in the processes of demyelination and also spontaneous remyelination, which initiates directly upon retrieval of the intoxicant, reviewed in (Kipp et al., 2009; Skripuletz et al., 2011; Vega-Riquer et al., 2019). These characteristics make this model ideal for testing regulatory properties of compounds specifically on adult oligodendroglial cell development and remyelination and preferred choice for the validation following screenings for promyelinating treatments (Cui et al., 2019; Deshmukh et al., 2013; Guo et al., 2018; Suo et al., 2019; Zhu et al., 2019).

Using this model, it was shown that the leading compounds parbendazole and danazol significantly accelerate OPC differentiation and spontaneous myelin repair upon cuprizone retrieval. In opposition to the clear-cut changes of parbendazole administration in oligodendroglial lineage progression along the midline of corpus callosum, danazol induced only a small significant increase in the number of GSTπ-positive cells. The less prominent effects of danazol can be explained by the fact that it was administered orally, which enables limited control over the consumed doses. Besides, this compound is characterized by poor

aqueous solubility and dissolution (Chen et al., 2004). To overcome this obstacle, a freshly prepared solution was applied daily, and the surfactant Tween 80 was added to the aqueous solution to increase stability. However, application of a higher concentration or a different surfactant, such as polyethylene glycol (PEG) esters of fatty acids instead could lead to effects that are more prominent. Alternatively, direct drug delivery into the stomach (via gastric gavage) may also be more efficient. Apart from substance solubility, another parameter taken into account during the design of these experiments was the predicted ability of both compounds to penetrate the blood-brain-barrier (BBB) according to the ADMET criteria (Dong et al., 2018). As this parameter is a determinant for the effectivity of a treatment, before a compound enters a clinical trial, BBB permeability should be specifically assessed to avoid false positive or negative results from this analysis. This can be done either *in vitro* (Kaisar et al., 2017), or in animal models with methods such as microdialysis (Bourne, 2003; Ducey et al., 2012) and autoradiography (Stumpf, 2012), followed by other studies for drug distribution in the CNS (X. Liu et al., 2006; Loryan et al., 2013). Moreover, regarding toxicity, parbendazole has been only reported to cause teratogenic effects in sheep and cattle at concentrations higher or equal to 60 mg/kg body weight (Szabo, 1989), which exceed the here applied concentration. Accordingly, danazol is an FDA approved treatment for endometriosis with only minor reported side effects (Dmowski et al., 1971). In this study, no cytotoxic effects have been investigated, however the positive effects on oligodendroglial cell development suggest otherwise. In addition, none of the animals included in this study showed obvious signs of suffering.

In these experiments, compound administration was initiated while microgliosis, astrogliosis, OPC proliferation, and oligodendrogenesis were still ongoing, with newly formed oligodendrocytes being vulnerable to cuprizone intoxication (Vega-Riquer et al., 2019). In addition, this study does not include determination of danazol- and parbendazole-induced effects on OPC proliferation or microglial and astrocyte activation, therefore, these effects along with cytoprotection cannot be fully excluded. However, upon comparison of the numbers of OLIG2-positive cells in substance and vehicle treated-mice significant differences

did not emerge (data not shown), thus speaking against a prominent impact on proliferation. Moreover, based on the here described *in vitro* and *ex vivo* data, this study suggests that the positive effects of these compounds on remyelination, are primarily induced via enhancement of OPC differentiation. Another aspect, which was not investigated in these experiments, were the effects of the treatments on neuroprotection. According to the current literature (Schultz et al., 2017; Zendedel et al., 2013) acute axonal injury is primarily taking place during active demyelination and only low levels can be detected at the time point of analysis in the traditional cuprizone set-up that has also been used in this study (6 weeks of cuprizone application and 1 week of remyelination). Similarly, absence of acute axonal injury was reported for remyelinated shadow plaques in MS patients and the chronic EAE animal model (Kornek et al., 2000). Hence, in order to describe to what degree compound treatment (positively affecting axonal remyelination) exerts an additional impact on axon integrity alternative experiments will have to be performed in future. To further elucidate the impact of these two leading compounds on regenerative processes upon demyelination, more elaborated analyses, including earlier time-points of analysis (i.e., 4, 5, and 6 weeks of cuprizone application) are required.

5.4. Biomedical translation process for multiple sclerosis treatment development

As previously explained cuprizone-evoked lesions resemble those of pattern III MS, while temporal aspects of demyelination and remyelination are also similar to MS pathology (Liu et al., 2010). However, the classic cuprizone-induced demyelination model, as used in this study, can be considered a useful model for the study of remyelination but no MS animal model, due to fundamental differences in pathology, mainly reflecting the absence of inflammatory cell infiltration from the periphery (Matsushima & Morell, 2001; Vega-Riquer et al., 2019).

One limitation of this model is that young animals are used, at an age which corresponds to a human age of below 18 years old, and therefore pathology similar to pediatric MS (Dutta & Trapp, 2014; Flurkey et al., 2007; Goodin, 2014) . In addition, remyelination in young mice is

progressing rapidly, thus hindering detection of myelin repair promoting activities (Lindner et al., 2009; Skripuletz et al., 2011). For these reasons, protocols for chronic cuprizone-mediated demyelination have been established using mice aged six months, a developmental stage which represents young adulthood in humans and especially around the age when MS symptoms usually begin (Dutta & Trapp, 2014; Flurkey et al., 2007; Goodin, 2014). In this chronic model, both the older age and the prolonged cuprizone application contribute to severe phenotypes which are followed by less prominent remyelination capacity and persisting pathological symptoms (Armstrong et al., 2006; Gingele et al., 2020; Lindner et al., 2009; Mason et al., 2004; Nomura et al., 2019). Chronic demyelination not only allows better study of oligodendendroglial cell regeneration and remyelination but also a more thorough determination of activities related to microglial and astrocytic activation, as well as acute axonal injury (Lindner et al., 2009). Extended remyelination after acute demyelination and inefficient remyelination in chronic lesions which allows neurodegeneration and permanent neurological deficits to develop are also characteristics of MS pathology (Lucchinetti et al., 1999; Ludwin, 1994; Prineas & Connell, 1979; Raine & Cross, 1989). Suggested etiology for the declined remyelination capacity during the course of the disease is the limited rate of oligodendrogenesis, which is caused due to the negative impact of aging (Doucette et al., 2010; Sim et al., 2002) and the lesion environment (Chang et al., 2000; John et al., 2002; Scolding et al., 1998; Wolswijk, 1998) on OPC proliferation and differentiation, the axonal damage (Raine & Cross, 1989; Silber & Sharief, 1999), and the inability of long-termed demyelinated axons to be remyelinated (Chang et al., 2002). Therefore, application of the lead compounds that emerged from this study in the chronic cuprizone demyelinating model, may enable determination of their effects in regulatory mechanisms that lead to diminished remyelination capacity in chronic MS lesions during disease progression and their impact on other glial cells and neurons.

Moreover, the use of the EAE rodent model for MS would allow investigation of the lead compounds in the context of immunomodulation and oligodendroglial cell development in an inflammatory environment which recapitulates the inflammatory MS lesion sites, reviewed in

(Denic et al., 2011; Robinson et al., 2014). The here presented study aimed to identify regenerative properties of chemical compound application. As the occurrence of demyelination and remyelination in EAE are not precisely defined, this model is not ideal for this purpose and therefore it was not chosen for validation of the hit compounds (Ransohoff, 2012). However, as variations of the experimental setup in EAE enable independent study of specific stages of MS progression such as relapsing–remitting EAE (Tuohy et al., 1989) and chronic progressive demyelination (Procaccini et al., 2015; Tompkins et al., 2002), a follow up study in this direction would increase the translational potential of our findings. In fact, as discussed earlier, there are reported studies where both non-immune driven, gliotoxin-mediated demyelination models and EAE were employed for a more detailed characterization of treatment-induced effects (Deshmukh et al., 2013; Najm et al., 2015; Suo et al., 2019). Interestingly, Najm et al. addressed this question regarding miconazole and clobetazol first with an LPC-induced *in vivo* model of demyelination and following with the relapsing-remitting $PLP_{139-151}$-induced EAE model and the MOG_{35-55}-induced EAE which induces a relative controlled immune response and chronic progressive demyelination, thus showing only remyelinating properties for miconazole and a combinatory positive effect with immunomodulation for clobetasol (Najm et al., 2015).

Another distinctive feature of MS pathology which determines drug distribution in the CNS and thus the efficacy of a treatment is the BBB breakdown, which is induced by inflammation (Grossman et al., 1986; Katz et al., 1993; Silver et al., 2001; Spencer et al., 2018). BBB permeability is not affected by non-inflammatory demyelination, therefore the cuprizone demyelination model is not addressing this aspect (Bakker & Ludwin, 1987). In EAE similar to MS, activated immune cells from the periphery induce BBB leakage (Balasa et al., 2020; Wolburg-Buchholz et al., 2009; Wolburg et al., 2004; Yang et al., 2016), hence using this animal model will enable determination of the administrated doses and compound distribution in CNS, as regulated by conditions that resemble the MS- affected brain.

Another pitfall of understanding MS pathology and developing regenerative approaches is the heterogeneity in the oligodendroglial cell populations in the adult brain (Foerster et al., 2019; Leong et al., 2014). Notably, using both tissue samples from EAE-mice and human MS-brains altered oligodendrocyte heterogeneity was reported when compared to the healthy counterparts, which can at least in part be explained as response to the inflammatory environment (Falcão et al., 2018; Jäkel et al., 2019). To further define the reasons for the inefficient remyelination in MS, more histological analyses using tissue from active/demyelinating MS lesions were performed, suggesting that oligodendroglial cell behavior and the progress of myelin sheath formation vary according to the type of lesion and even the exact location within the lesion due to the hostile environment and the extent of the demyelinating episode (Brück et al., 1994; Heß et al., 2020). In addition, as mentioned before there are phenotypic differences between human and rodent OPCs which cannot be overseen (Jäkel et al., 2019; Perlman et al., 2020). Taken these impediments into account, investigation of drug-induced effects in MS tissue may prevent unmet goals of clinical trials later during drug development. As broad compound testing in MS tissue is practically not feasible, one possibility to this goal is to investigate the role of known lead compound targeted mechanisms in lesion development, oligodendrogenesis and myelin development using MS tissue. A bright example in this regard comes from our research group, where upon showing a positive regulatory role of the CXCL12 receptor CXCR7 in OPC differentiation in the inflamed rat brain (Göttle et al., 2010), the effects were validated using human fetal OPC cultures and in addition, its expression in MS remyelinating lesions was detected (Kremer et al., 2016b). The differential expression of CXCR7 in different types of MS lesions strongly suggests implication of the regulatory mechanism in remyelination in the context of the disease, thus suggesting a prominent therapeutic potential of its pharmacological modulation for myelin repair (Kremer et al., 2016b).

5.5. Drug repurposing and hit compound target identification

Drug target identification is a critical step in drug development and contributes to the investigation of molecular and cellular mechanisms implicated in several pathologies (Núñez

et al., 2012). Various databases of known targets and computational models for potential target predictions facilitate this process with low requirements in terms of time and cost (Chen et al., 2016). In this study confirmed primary hits were assigned into categories according to the most prominent potential targets (Fig. 1d), upon interrogation of the databases DrugBank (Law et al., 2014), STITCH (Kuhn et al., 2014), ChEMBL (Gaulton et al., 2017) and the web server SwissTargetPrediction (Gfeller et al., 2014). Interestingly, in most cases molecular mechanisms implicated in oligodendroglial cell development were emerged as known or potential targets. Despite the significant information these databases and computational models offer, false predictions and off-targets (Chartier et al., 2017) complicate target identification. To overcome these difficulties extensive literature research in the context of the study and experimental work are required.

In this respect, literature research was conducted for the validated hits methiazole, parbendazole, nocodazole and danazol. The first three belong to the compound family called benzimidazoles, which has been related to anti-cancer, anti-protozoal, anti-helminthic, anti-inflammatory, anti-microbial, anti-viral, and analgesic activities (Salahuddin et al., 2017). Most of them are used in veterinary medicine where in some cases teratogenic or cytotoxic effects have been reported (Radostits, 2007), while others, such as the anti-ulcer treatments pantoprazole and omeprazole are broadly used in human medicine without significant implication of adverse effects. Methiazole’s cell cycle profile linked this compound with parbendazole and two other microtubule destabilizing agents (Lo et al., 2017), therefore suggesting effects mediated via similar mechanisms. Specifically, based on the observation that parbendazole stimulation induces phosphorylated p38MAPK protein in rat primary OPC culture, it is suggested here that both parbendazole and methiazole induce alterations in microtubule dynamics via a p38MAPK-related mechanism. In line with these findings, similar phosphorylation events were previously reported in response to omeprazole stimulation (Zhu et al., 2019). Acute nocodazole stimulation at nanomolar concentrations, in contrast, was shown to promote OPC maturation via increased microtubule arborization, while at micromolar levels increased cytotoxicity and adverse effects during *in vitro* myelination were

observed (Lee & Hur, 2020). This reported nocodazole-induced toxicity, is in accordance with the here reported findings and advocates the exclusion from further validation in this study.

Danazol is a testosterone derivative, initially approved by the FDA as treatment for endometriosis, which has reported anti-gonadotropic and anti-estrogenic properties (Dmowski et al., 1971). As danazol is a largely studied and approved drug, retrieving information about its mode of action and application both from databases (e.g., DrugBank) and the literature is much easier than for the benzimidazole derivatives. In fact, it has been studied in cancer research for its cytotoxic properties against, among others, leukemic and multidrug-resistant cancer cells (Chang et al., 2019; Podhorecka et al., 2016). The only reported effect of this compound in the context of oligodendoglial cell biology prior to this study was rescue of MBP expression in a zebrafish mutant featuring partial or complete loss of MBP transcripts in the peripheral nervous system (Diamantopoulou et al., 2019). However, the findings of this study are supported by the fact that other steroids are known to induce myelin repair activities. Precisely, progesterone and nestorone were shown to improve oligodendroglial cell maturation and remyelination a chronic model of cuprizone mediated demyelination (El-Etr et al., 2015). Moreover, other studies demonstrated remyelination promoting properties of testosterone and its synthetic analogue 7α-methyl-19-nortestosterone treatment in both chronic and acute demyelinated lesions via androgen receptor activation (Bielecki et al., 2016; Hussain et al., 2013).

The here presented study aimed to elucidate the underlying mechanism of p57kip2's nuclear exclusion and the mediation of its regulatory impact on oligodendroglial cell differentiation. Previous work from our research group has brought to light regulatory mechanisms of the intracellular p57kip2 translocation related to gene expression (via Ascl1 and Hes5), cytoskeletal dynamics (via LIMK-1), cell cycle exit [CDK2; (Göttle et al., 2015)] as well as vascular ATPase activity (Göttle et al., 2019), however, the exact cascades are not yet known. Predicted/known targets of the primary hits emerged from the screening conducted for the purpose of this study, suggest that extrinsic signals targeting ion channels (benzamil

hydrochloride) and neurotransmitter receptors (alverine citrate salt), microtubule dynamics (benzimidazoles), steroid hormone receptors (danazol), COX1/2, and STAT5 (isoxicam and fosfosal) may be involved in the p57kip2 shuttling during OPC differentiation (Fig. 1d). As parbendazole showed the most prominent pro-differentiation and myelin repair effects a deeper look at the underlying mechanism was taken. Previous observations illustrating that CXCL12 chemokine stimulation induces MAPK phosphorylation and OPC differentiation (Göttle et al., 2010) are in accordance with the experimental data regarding parbendazole mediated p38MAPK activation in this study. In addition, p57kip2's nucleocytoplasmatic shuttling in differentiating oligodendrocytes has been shown to be mediated through the exporting 1 [CRM1, (Göttle et al., 2015)]. Interestingly, p38MAPK activation has been associated with nuclear transport of several proteins such as MAPK-activated protein kinase 5 (MK5), NFAT and E2F1, in all cases in a CRM1 dependent manner (del Arco et al., 2000; Ivanova & Dagnino, 2007; Seternes et al., 2002). Taken together, more extensive study of parbendazole mediated p38MAPK activation may shed light to the underlying mechanism of p57kip2 nuclear shuttling.

To further investigate the effects on oligodendroglial cell development induced by danazol and benzimidazole derivate treatment, bioinformatic data are currently being analyzed. Specifically, available differential gene expression profiles in oligodendroglial cells resulted from danazol and a microtubule destabilizing agent administration in mice, are being evaluated using Gene Ontology (GO) Term Enrichment using PANTHER Gene List Analysis tools (Mi et al., 2019). Once specific clusters of genes related to molecular processes relevant to OPC differentiation and remyelination are identified to be regulated, transcriptional changes will be investigated in the context of remyelination following cuprizone mediated demyelination. To this goal, using the same experimental setup and compound administration ways, RNA samples from the area along the midline of the medial and caudal compartments of the corpus callosum (where the highest levels of demyelination were detected by means of immunohistochemistry) will be extracted and investigated via quantitative polymerase chain reaction (qPCR).

5.6. Conclusion

The present thesis suggests the subcellular localization of a potent inhibitor of oligodendroglial cell differentiation, namely p57kip2 protein, as differentiation competence marker. Using this readout, a phenotypic compound screening for small molecules with the potential to promote OPC differentiation was established and successfully performed. During this screening 21 primary hits were found to induce nuclear exclusion of the protein and among them methiazole, parbendazole, and danazol were found to promote differentiation of both rat and human primary OPCs, as well as *ex vivo* myelination in the rat system. Parbendazole and danazol enhanced OPC differentiation and remyelination upon toxin mediated demyelination in mice. Given that different biological systems (rat, mouse and human) and brain areas (cerebral cortex, cerebellum, corpus callosum) were included in this study, these data suggest evolutionary conserved molecular targets, thus highlighting their biomedical translational potential.

These results suggest parbendazole and danazol as lead compounds for optimization later in the process of drug development for the treatment of MS. Their effects in an inflammatory background or in other cell types in the CNS remain to be shown. In addition, known or predicted targets of all primary hits can be used to shed light to the underlying mechanism of p57kip2 translocation. Of great interest would be a follow up study of the p38MAPK related effects observed upon parbendazole stimulation and elucidation of a possible link to the nuclear exclusion of the p57kip2 protein. Finally, investigation of the differential gene expression during remyelination in the cuprizone model induced by parbendazole and danazol will provide more concrete information about their molecular targets.

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7. LIST OF ABBREVIATIONS

µl microliter

µm micrometer

µM micromolar

5-HAT 5-hydroxytryptamine

Ace acetopromazine maleate salt

ADHD attention deficit hyperactivity disorder

AEP anterior entopeduncular area

ALS amyotrophic lateral sclerosis

Alv alverine citrate salt

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APC adenomatous polyposis coli protein

ASCL1/MASH1achaete-scute homolog 1

ATX autotaxin

BBB blood brain barrier

BDNF neurotrophin brain-derived neurotrophic factor

Ben benzamil hydrochloride

BMP bone morphogenetic protein

BRG1 brahma-related 1 complex

BSA Bovine Albumin Fraction V

CC1 adenomatous polyposis coli protein (APC) clone CC1

CDK cyclin-dependent kinase

CDKI cyclin-dependent kinase inhibitor

CGE caudal ganglionic eminence

Cis cisapride

CMAP connectivity map

CNP 2'3'-cyclic nucleotide 3'-phosphodiesterase

CNS central nervous system

CPZ cuprizone

CRM1 exportin 1

CSF cerebrospinal fluid

CSPG chondroitin sulfate proteoglycan

CSPG4chondroitin sulfate proteoglycan type 4

Dan danazol

DAPI 4',6-diamidino-2-phenylindole

DHA docosahexaenoic acid

DMEM Dulbecco’s modified eagle medium

DMSO dimethylsulfoxide

DNMT DNA methyltransferase

Dox doxepin hydrochloride

EAE experimental autoimmune encephalomyelitis

EC50 half maximal effective concentration

ECM extracellular matrix

EMA Europeans Medicines Agency

EpiSC pluripotent mouse epiblast stem cell

FBS fetal bovine serum

FDA US Food and Drug Administration

Fel felodipine

FGF fibroblast growth factor

Fig. figure

Flu fluvoxamine maleate

Fos fosfosal

GABA gamma-Aminobutyric acid

GALC galactocerebrosidase

GalC galactocerebroside

GO gene ontology

GPCR G protein-coupled receptor

GR glucocorticoid receptor

GSK3 glycogen synthase kinase 3

GST glutathione-S-transferase

GST-pi glutathione-S-transferase pi cytoplasmic isoform

h hour

H3K9 histone H3 lysine 9

H3R histamine receptor-3

HA hyaluronan

HAT Histone acetyltransferase

HBSS Hank's balanced salt solution

HDAC histone deacetylase

hESC human embryonic stem cell

HTS high-throughput screening

i.p. intraperitoneally

IGF insulin-like growth factor

iPSC induced pluripotent stem cell

Iso isoxicam

kDA kilodalton

kg kilogram

KOR Kappa opioid receptor

LANUV Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen

LGE lateral ganglionic eminence

LIF leukemia inhibitory factor

LIMK-1 LIM kinase-1

LINGO-1 leucine-rich repeat and Ig domain-containing, Nogo receptor-interacting protein

LncRNA long noncoding RNAs

LPC lysophosphatidylcholine

LXR liver X receptor

MAG myelin-associated glycoprotein

MBP myelin basic protein

MEM minimum essential medium

Met methiazole

mg milligram

MGE medial ganglionic eminence

MHV mouse hepatitis virus

Mia mianserine hydrochloride

min minute

ml milliliter

mM millimolar

MOBP myelin-associated oligodendrocyte basic protein

MOG myelin oligodendrocyte glycoprotein

MOR mu opioid receptor

MR mineralocorticoid receptor

MS multiple sclerosis

n number of experiments

NCAM neural cell adhesion molecule

ncRNA noncoding RNAs

NF neurofilament

NG2 neuron-glial antigen-2

NgR1 Nogo-66 receptor

NIH National Institutes of Health

NLS nuclear localization sequence

NMDA N-methyl-d-aspartate

NMO neuromyelitis optica

Noc nocodazole

OPC oligodendroglial precursor cell

Opi opipramol dihydrochloride

Par parbendazole

PBS Dulbecco's phosphate buffered saline

PDE7 phosphodiesterase 7

PDGF platelet-derived growth factor

PDL poly-D-lysine

PEG polyethylene glycol

Pen pentamidine isethionate

PFA paraformaldehyde

Piz pizotifen malate

PLP proteolipid protein

PMD Pelizaeus-Merzbacher disease

pMN motor neuron progenitor domain

PNS peripheral nervous system

PPARγ peroxisome proliferator-activated receptor gamma

PPMS primary progressive MS

PRMT protein arginine methyltransferase

PSA polysialic acid

QKI RNA-binding protein Quaking

qPCR quantitative polymerase chain reaction

ROCK Rho-associated kinase

rpm revolutions per minute

RRMS relapsing-remitting MS

RT room temperature

RxRγ Retinoic x Receptor gamma

S1P Sphingosine-1-Phosphate

S1PR sphingosine 1-phosphate receptor

SCAP SREBP cleavage activating protein

SDS sodium dodecyl sulfate

sec second

SEM standard error of the mean

Shh Sonic hedgehog

SPMS secondary progressive MS

SREBPsterol responsive element binding protein

TBS Tris-Buffered Saline

TET ten-eleven translocation

TMEV Theiler's murine encephalomyelitis virus

TNFa tumor necrosis factor a

Tol tolcapone

veh vehicle

VZ ventricular zone

YY1 Yin Yang 1

ZETT Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben

8. ANNEX

8.1. Published research article

Parts of this thesis are based on a published manuscript. According to the paragraph § 2 (4) of the doctoral regulations of the faculty of mathematics and natural sciences of Heinrich Heine University Düsseldorf of 15.06.2018, such parts of the manuscript which have been reproduced or adapted are clearly marked in the thesis.

Complete reference to the manuscript:

Manousi, A., Göttle, P., Reiche, L., Cui, Q.-L., Healy, L. M., Akkermann, R., Gruchot, J., Schira-Heinen, J., Antel, J. P., Hartung, H.-P., & Küry, P. (2021). Identification of novel myelin repair drugs by modulation of oligodendroglial differentiation competence. EBioMedicine, 65, 103276. https://doi.org/10.1016/j.ebiom.2021.103276

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Anastasia Manousi (Doctoral candidate), Peter Göttle, Laura Reiche, Qiao-Ling Cui, Luke M. Healy, Rainer Akkermann, Joel Gruchot, Jessica Schira-Heinen, Jack P. Antel, Hans-Peter Hartunga, Patrick Küry.

Contribution of the doctoral researcher to the content of the manuscript: approx. 75%

Anastasia Manousi contributed to methodology, software design, validation, formal analysis, investigation, data curation, writing-original draft, writing – review & editing, visualization, funding acquisition, data verification.

The writing of the original draft, as well as the review and the editing were conducted with consultation and supervision from Prof. Dr. Patrick Küry, and figure design in collaboration with Dr. Peter Göttle.

Experiments with human fetal brain-derived OPCs were conducted and analyzed by our collaborators Dr. Qiao-Ling Cui, Dr. Luke M. Healy and Prof. Dr. Jack P. Antel, in the

Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC H4A 3K9, Canada.

Western blot experiments were conducted and analyzed in our laboratory by Laura Reiche with consultancy from Dr. Jessica Schira-Heinen and Anastasia Manousi.

Dr. Rainer Akkermann and Joel Gruchot significantly contributed to the establishment of the *in vivo* cuprizone model.

Throughout this study Anastasia Manousi was offered technical assistance from Brigida Ziegler and Birgit Blomenkamp, while Dr. Michael Dietrich contributed with scientific consultancy and assistance to the handling of the BD Pathway 855 High-Content Cell Analyzer.

Prof. Dr. Patrick Küry and Dr. Peter Göttle conceptualized the project and Prof. Dr. Patrick Küry was responsible for the supervision of the study.

8.2. Manuscript in preparation

Many theoretical parts of this study (not marked) were used for the preparation of a literature review article entitled as "Pharmacologically active compounds at the service of myelin repair" authored by Anastasia Manousi and Prof. Dr. Küry. This article is not yet submitted.

8.3. Full western blots

Presentation of the full western blots used for the generation of the data presented in Fig. 10, as published in (Manousi et al., 2021). Evaluation of the efficiency of protein transfer following SDS electrophoresis was conducted using Pierce Reversible Protein Stain (Step 1). All staining procedures were performed in lanes 3 and 4 in a sequential manner (Steps 2-4) upon stripping of the same membrane. The areas of interest which were used for normalization to the total amount of the loaded protein are indicated with a red frame. MM refers to MagicMarkTM XP.

Step 1

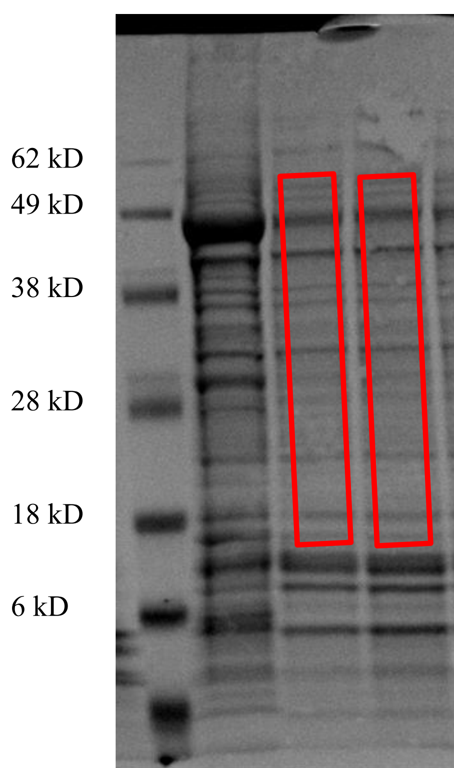
SeeBlue® Pre-Stained Protein Standard +MagicMark™ XP

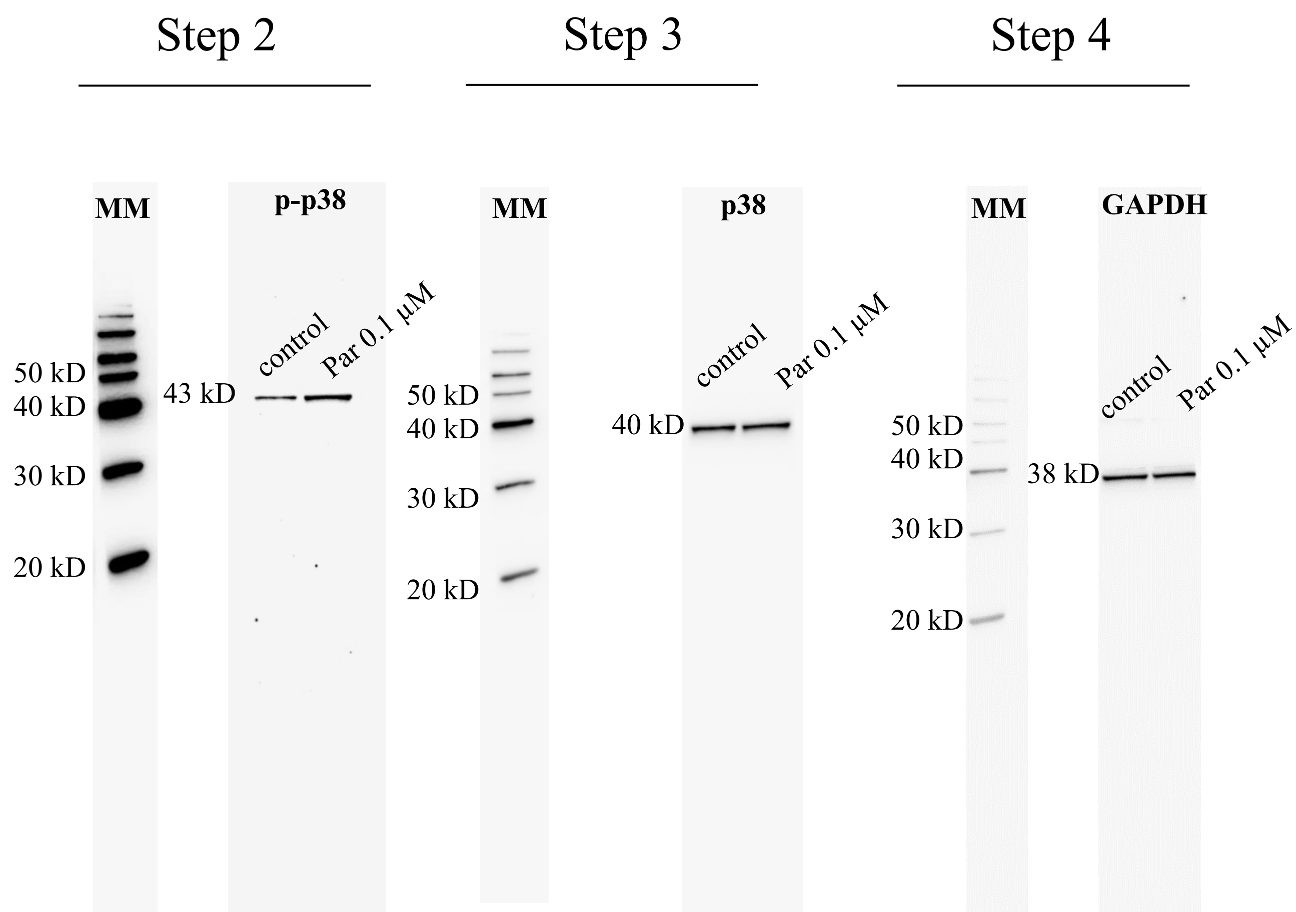
Whole brain lysate 20µg

Control 50µg

Par 0.1 µM 50µg

Lanes 1 2 3 4




Step 2
Step 3
Step 4
MM
p-p38
control
Par 0.1 μM
50 kD
40 kD
30 kD
20 kD
43 kD
MM
p38
control
Par 0.1 μM
50 kD
40 kD
30 kD
20 kD
40 kD
MM
GAPDH
control
Par 0.1 μM
50 kD
40 kD
30 kD
20 kD
38 kD

8.4. Negative results (unpublished data)

8.4.1. Validation of primary hits

In the table below is presented the complete unpublished data set from three validation rounds of the primary hits. All compounds were tested and analyzed as presented in paragraph 4.1. The data are presented in comparison to the negative [differentiation medium (DM) + DMSO] and positive controls (Fingolimod 1 µM) and as mean values.

Compound	% Cells with nuclear p57kip2 DM + DMSO	% Cells with nuclear p57kip2 Fingolimod	% Cells with nuclear p57kip2 Compound
Mebeverine hydrochloride	76.94	52.68	51.86
Ifenprodil	76.94	52.68	57,71
Halcinonide	76.94	52.68	63.51
Benzamil hydrochloride	76.94	52.68	36.44
Opipramol dihydrochloride	76.94	52.68	57.62
Biotin	76.94	52.68	69.21
Felodipine	76.94	52.68	51.57
Alverine citrate salt	76.94	52.68	53,38
Methocarbamol	76.94	52.68	65.49
Hexamethonium dibromide dihydrate	76.94	52.68	57.74
Diflunisal	76.94	52.68	65.52
Isoxicam	76.94	52.68	57.17
Sulfamethoxypyridazin	76.94	52.68	62.58
Azaguanine-8	74.25	60.30	70.82
Ethambutol dihydrochloride	74.25	60.30	69.83
Propafenone hydrochloride	74.25	60.30	64.68
Norcyclobenzaprine	74.25	60.30	74.50
Pentamidine isethionate	74.25	60.30	45.15

Lansoprazole	74.25	60.30	67.00
Methiazole	74.25	60.30	54.28
(+,-)-Synephrine	74.25	60.30	72.99
Denatonium benzoate	74.25	60.30	70.19
Norgestimate	74.25	60.30	Partial cytotoxicity
Pizotifen malate	74.25	60.30	55.97
Fluvoxamine maleate	74.25	60.30	55.01
Acyclovir	75.71	56.16	66.67
Mianserine hydrochloride	75.71	56.16	57.01
Cilnidipine	75.71	56.16	Partial toxicity
Danazol	75.71	56.16	36.65
Aminohippuric acid	75.71	56.16	61.28
Acetopromazine maleate salt	75.71	56.16	49.41
Furaltadone hydrochloride	75.71	56.16	73.08
Guaifenesin	75.71	56.16	66.91
Bendroflumethiazide	75.71	56.16	68.30
Propidium iodide	75.71	56.16	90.72
Methotrimeprazine maleat salt	75.71	56.16	72.44
Fosfosal	75.71	56.16	56.98
Leflunomide	74.25	60.30	60.61
Tolcapone	75.71	56.16	52.92
Nocodazole	68.66	38.60	48.19
Doxepine hydrochloride	68.66	38.60	46.97
Clomipramine hydrochloride	68.66	38.60	Partial cytotoxicity
Vincamine	68.66	38.60	60.30
Ketoconazole	68.66	38.60	55.25
Fusidic acid sodium salt	68.66	38.60	62.69

Cisapride	68.66	38.60	50.59
Thiorphan	68.66	38.60	53.11
Oxybenzone	68.66	38.60	57.08
Hymecromone	68.66	38.60	62.99
Methyldopate hydrochloride	68.66	38.60	Partial cytotoxicity
Parbendazole	68.66	38.60	48.94

8.4.2. Secondary screening

In the table below are presented all the unpublished results from the secondary screening. All the compounds were applied at concentrations 10, 5, and 1 µM for three days and analyzed as presented in the paragraph 4.2. Selected compounds were tested at concentrations 0.1, 0.02, and 0.01 µM. The compounds ifenprodil, hexamethonium dibromide dihydrate, and mebeverine hydrocloride were not subjected to validation due to technical issues. The data are presented in comparison to the control (DMSO at the corresponding concentration) and as mean values and standard error of the mean (SEM). None of the here presented results are statistically significant.

Compound/concentration	**Number of experiments**	**% MBP+ cells Compound/SEM**	**% MBP+ cells Control/SEM**
Fluvoxamine maleate/10 µM	1	Extensive cytotoxicity	
Fluvoxamine maleate/5 µM	4	21.52/6.74	21.51/6.55
Fluvoxamine maleate/1 µM	3	24.25/4.03	21.59/4.06
Fluvoxamine maleate/0.1 µM	6	22.18/3.56	22.11/3.76
Acetopromazine maleate salt/5 µM	1	Extensive cytotoxicity	
Acetopromazine maleate salt/5 µM	1	29.26/6.46	29.36/6.12
Acetopromazine maleate salt/1 µM	2	23.10/5.06	27.64/4.82
Acetopromazine maleate salt/0.1 µM	1	27.87/10.62	24.72/7.12
Mianserine hydrochloride/10	1	Extensive	

μM		cytotoxicity	
Mianserine hydrochloride/5 μM	1	16.76/9.44	24.72/7.12
Mianserine hydrochloride/1 μM	2	25.13/7.16	25.94/5.00
Mianserine hydrochloride/0.1 μM	2	25.10/5.26	23.89/5.03
Cisapride/10 μM	1	Extensive cytotoxicity	
Cisapride/5 μM	3	24.30/3.36	24.30/4.30
Cisapride/1 μM	4	22.99/2.81	22.18/3.55
Cisapride/0.1 μM	4	23.82/3.16	21.74/3.40
Tolcapone/10 μM	1	32.69/8.17	25.96/6.38
Tolcapone/5 μM	2	28.10/5.28	28.74/5.12
Tolcapone/1 μM	1	24.42/8.48	27.15/7.01
Tolcapone/0.1 μM	1	24.05/9.72	23.06/7.11
Fosfosal/10 μM	1	30.32/7.22	32.69/8.17
Fosfosal/5 μM	1	27.00/4.11	28.74/5.12
Fosfosal/1 μM	2	25.33/7.78	29.92/5.37
Fosfosal/0.1 μM	2	24.26/4.32	23.06/7.11
Doxepine hydrochloride/10 μM	1	Extensive cytotoxicity	
Doxepine hydrochloride/5 μM	3	25.77/4.95	25.80/5.27
Doxepine hydrochloride/1 μM	3	26.12/6.53	26.22/5.28
Doxepine hydrochloride/0.1 μM	2	26.48/5.07	28.48/7.59
Pizotifen malate/10 μM	1	Extensive cytotoxicity	
Pizotifen malate/5 μM	1	5.82/4.36	21.97/7.80
Pizotifen malate/1 μM	3	26.44/4.21	24.97/4.74
Pizotifen malate/0.1 μM	4	26.15/2.60	25.03/3.82
Pentamidine isethionate/10 μM	1	Extensive cytotoxicity	
Pentamidine isethionate/5 μM	1	Extensive cytotoxicity	
Pentamidine isethionate/1 μM	3	24.15/3.62	23.76/4.40
Pentamidine isethionate/0.1 μM	3	25.54/4.46	24.35/4.10
Methiazole/10 μM	1	Extensive	

		cytotoxicity	
Methiazole/5 µM	2	44.10/10.60	26.94/7.83
Methiazole/1 µM	3	32.30/6.67	25.08/6.23
Parbendazole/10 µM	1	Extensive cytotoxicity	
Parbendazole/5 µM	1	Extensive cytotoxicity	
Isoxicam/10 µM	1	Extensive cytotoxicity	
Isoxicam/5 µM	1	Extensive cytotoxicity	
Fosfosal/10 µM	1	Extensive cytotoxicity	
Fosfosal/5 µM	1	Extensive cytotoxicity	
Nocodazole/5 µM	1	Extensive cytotoxicity	
Nocodazole/1 µM	1	Extensive cytotoxicity	
Nocodazole/0.01 µM	3	24.58/4.99	21.79/3.83
Danazol/10 µM	1	Extensive cytotoxicity	
Danazol/1 µM	3	22.34/3.23	25.46/2.56
Benzamil hydrochloride/10 µM	2	6.55/1.33	10.40/3.84
Benzamil hydrochloride/5 µM	2	15.02/4.20	12.99/3.30
Benzamil hydrochloride/1 µM	4	20.32/7.46	13.69/3.66
Benzamil hydrochloride/1 µM	4	20.32/7.46	13.69/3.66
Benzamil hydrochloride/0.1 µM	3	25.89/6.43	22.90/3.70
Felodipine/10 µM	1	Extensive cytotoxicity	
Felodipine/5 µM	1	Extensive cytotoxicity	
Felodipine/1 µM	4	Extensive cytotoxicity	

Felodipine/0.1 μM	3	13.85/6.74	14.55/6.04
Felodipine/0.01 μM	3	14.02/5.12	14.23/3.24
Alverine citrate salt/10 μM	1	Extensive cytotoxicity	
Alverine citrate salt/5 μM	1	Extensive cytotoxicity	
Alverine citrate salt/1 μM	1	Extensive cytotoxicity	
Alverine citrate salt/0.1 μM	3	19.56/4.81	14.55/6.04
Alverine citrate salt/0.01 μM	2	17.77/7.22	14.22/3.43

8.4.3. Validation in terms of *ex vivo* developmental myelination

In the table below are presented the unpublished results for the non-optimal compound concentrations applied on organotypic brain slices. Compound treatment and analysis were performed as presented in paragraph 4.4. The data are presented in comparison to the control (DMSO at the corresponding concentration) and as mean values and standard error of the mean (SEM). The here presented results are statistically significant.

Compound/concentration	**Number of experiments**	**% myelinating/ OLIG2+ cells Compound/SEM**	**% myelinating/ OLIG2+ cells Control/SEM**
Methiazole/1 μM	3	33.96/4.50	37.31/4.39
Danazol/5 μM	3	35.43/4.80	35.12/4.31

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10. DECLARATION OF AUTHORSHIP

I declare under oath that I have produced my thesis independently and without any undue assistance by third parties under consideration of the ‘Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf.

Anastasia Manousi, Düsseldorf, 21.07.2021

Name, Signature Place, Date