



Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Targeting the Sphingosine 1-Phosphate Axis as a Therapeutic Strategy in Krabbe's Disease: A Pre-Clinical Study

**This thesis submitted to University of Dublin, Trinity College
for the degree of
Doctor of Philosophy**

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19th March 2020

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Declaration and Statement of Plagiarism

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Acknowledgments

First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Kumlesh Dev, for the continuous mentorship, insightful guidance and encouragement for independent work. His expertise and patience guided me at all levels of my research project. He has provided boundless support on both academic and personal levels.

I am genuinely grateful to Dr. Steven Fagan for their assistance throughout the project. I would like to acknowledge the work of Dr. Sinead O'Sullivan and Justin Yssel who contributed to collecting behavioural parameters of the study.

I would also like to thank the current and former lab members for their support, scientific collaboration and friendship: Danai Stefanaki, Cedric Misslin, Luke Davison, Trisha Ang, Dr. Kapil Sharma and, most of all, Dr. Maria Velasco. I am deeply grateful to all the members of the Department of Physiology for their hospitality and assistance, in particular Mr. Johnny Fitzpatrick. I am also grateful to Dr. Gavin McManus for his help with confocal microscopy and IMARIS analysis.

Finally, I would like to thank my family and friends for their endless support throughout my academic career.

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List of Abbreviations

AAV	adeno-associated viral	ML	molecular layer
BAF312	Siponimod	MMP3	Matrix metalloproteinase 3
BBB	Blood brain barrier	MOG	Myelin Oligodendrocyte Protein
BMT	Bone marrow therapy	MPZ	Myelin Protein Zero
BSA	Bovine serum albumin	MS	Multiple Sclerosis
CGT	ceramide galactosyl transferase	NFH	Neurofilament Heavy
CNS	Central nervous system	OCT	Optimal cutting temperature
EAE	Experimental autoimmune encephalomyelitis	OFM	Open Field Maze
EDG	Endothelial differentiation gene	OPC	Oligodendrocyte progenitor cell
EMA	European Medicines Agency	PAS	Periodic Acid Schiff
ERT	Enzyme replacement therapy	PBS	Phosphate-buffered saline
FDA	Food and Drug Administration	PCL	Purkinje cell layer
FTY720	Fingolimod	pFTY720	phosphorylated FTY720
GALC	Galactosylceramidase	PGD2	Prostaglandin D2
GFAP	Glial fibrillary acidic protein	PKC	Protein Kinase C
GL	Granular layer	PLA2	Phospholipase A2
GPCR	G-protein coupled receptor	PND	Postnatal day
HPRA	Health Products Regulatory Authority	PNS	Peripheral nervous system
HSCT	Hematopoietic stem cell transplantation	RIPA	Radioimmunoprecipitation assay
Iba1	Ionized calcium binding adaptor molecule 1	RRMS	Relapsing-remitting Multiple Sclerosis
IHC	Immunohistochemistry	S1P	Sphingosine 1-phosphate
iNOS	induced nitric oxide synthase	S1PR	Sphingosine 1-phosphate receptor
KD	Krabbe's disease	SEM	Standard error of the mean
LFB	Luxol fast blue	SphK	Sphingosine kinase
M6P	mannose-6-phosphate	TNF	Tumour necrosis factor
MBP	Myelin Basic Protein	WM	white matter

Scientific Abstract

Krabbe's disease (KD, globoid cell leukodystrophy) is a rare infantile neurodegenerative disorder, caused by the defective gene *galc* encoding the lysosomal hydrolase galactosylceramidase (GALC). A subsequent decreased enzymatic activity leads to the accumulation of the toxic metabolite galactosylsphingosine (psychosine). Supra-physiological levels of psychosine aggregates, in turn, cause profound demyelination and severe loss of oligodendrocytes, usually accompanied by neuroinflammation. Currently clinical practice lacks a curative treatment and is mostly directed towards symptomatic relief. Recent findings highlight the sphingosine 1-phosphate (S1P) axis as a therapeutic target in multiple sclerosis. Our lab has previously shown that S1P receptor modulation with fingolimod and siponimod, two S1P receptor agonists, attenuate psychosine-induced cell death of human astrocytes *in vitro* and demyelination in mouse organotypic cerebellar slice cultures. Together, this data prompted the current preclinical studies investigating the effects of fingolimod in both adolescent and neonatal twitcher mice, the murine model of KD (**Results, chapter 3 and 4**). We additionally examined the relative impact of S1PR₁, S1PR₃ and S1PR₅ subtypes by administering the selective S1PR_{1/5} agonist, siponimod (**Results, chapter 5**). Using this S1PR₃ sparing compound we investigated the role and importance of S1PR₃ in the context of demyelination and inflammation in twitcher mice. Here, we found that fingolimod, but not siponimod, administered at postnatal day (PND) 21 resulted in improved myelination in twitcher mice (**Results, chapter 3 and 5**). Such results raise the hypothesis that remyelination may be, in part, linked to S1PR₃ activation. Interestingly, both compounds, however, were able to alter microglia and astrocytes activation, thus suggesting that S1PR_{1/5} was sufficient to modulate the activation state of these cells. Neonatal administration of fingolimod was able to reduce myelin debris, dampen microglial activation, without, however, increasing myelin markers (**Results, chapter 4**). Behaviourally, fingolimod was able to modestly enhance lifespan, with sporadic phenotypic improvements, when administered at PND5 and PND21, whereas siponimod did not result in a significant increase in lifespan. Although both compounds were able to provide partial efficacy within the CNS, they failed to improve peripheral systems (sciatic nerve, kidney, spleen). Such findings emphasise the importance of combination therapies targeting both the CNS as well as peripheral systems in a multimodal illness such as KD.

Lay Abstract

Krabbe's disease is a disorder of the brain that affects children within the first few months of life. It is caused by a deficiency in an enzyme, which leads to the build-up of a toxic substance called psychosine. Psychosine is damaging to the different cell-types within the brain. There are four main types of cells in the brain: (i) nerve cells, which process information via electrical signals, (ii) oligodendrocytes, which form a protective layer (myelin) around nerve cells, (iii) microglia, which provide immune defence within the brain, and (iv) astrocytes that communicate with other cells, provide support and ensure proper functioning of nerve cells. The toxic accumulation of psychosine in the brain of children with KD will negatively affect all of those cells. Two drugs that have been developed for the treatment of multiple sclerosis, can act on the brain cells to protect them from damage. We therefore used these two drugs, fingolimod and siponimod, in this study to examine whether they could be beneficial for the treatment of KD. Using an animal model of KD, the twitcher mouse, we analysed the effect of both compounds on the brain and clinical symptoms. We found that fingolimod prevented myelin, the insulating sheath surrounding nerve cells, from degenerating (demyelination). While siponimod did not prevent demyelination, it was able to reduce activation of immune cells. Both compounds had limited effects within the rest of the body, and multi-organ failure ultimately resulted in the death of the twitcher mice. Our study suggests that treatment of KD could benefit from drugs such as fingolimod and siponimod, if they are administered in conjunction with other compounds that specifically prevent the multi-organ failure observed in KD.

Aims and Hypothesis

The aims of the study are as follows:

- Determine whether fingolimod- and siponimod-mediated effects are protective against demyelination observed in the CNS and PNS of twitcher mice.
- Investigate if S1PR modulation may be neuroprotective and/or induce neurogenesis.
- Investigate the role of S1PR modulation in regulating astrocyte and microglial activation following fingolimod and siponimod administration in twitcher mice.
- Evaluate potential changes in PAS-positive globoid cell formation following fingolimod administration in twitcher mice.
- Examine whether fingolimod results in adverse events when administered during weaning period and how it affects early developmental stages.
- Investigate whether beneficial fingolimod-mediated effects on above mentioned outcome measures in twitcher mice can be further enhanced when administered pre-symptomatically.
- To examine the relative importance of S1PR₁, S1PR₃ and S1PR₅ modulation in demyelination and inflammation.
- To assess behavioural outcomes including lifespan, ataxia, immobility and weight loss, of fingolimod- and siponimod-treated twitcher mice compared to vehicle (water)-treated twitcher and wildtype animals.

We hypothesise that S1PR modulation with fingolimod will result in protective effects in twitcher mice. We expect S1PR modulation to mediate remyelination and dampen the immune processes in twitcher mice. Based on previously published findings (**O'Sullivan et al. 2016**), we also hypothesise siponimod to act in a similar manner to fingolimod, resulting in improved myelination and inflammation in twitcher mice. We expect such improved myelination to reflect positively in our behavioural outcome measures, with increases in lifespan and phenotypic abnormalities.

Value of Research

Krabbe's Disease (KD) is a rare, infantile degenerative disorder, impairing the development of myelin sheath with severe detrimental consequences on mental and motor skills. The leading hypothesis explaining the pathogenesis of KD is based on the loss of oligodendrocytes due to psychosine-induced cytotoxicity. However, its exact mechanisms of action and intracellular signalling pathways remains to be elucidated. It is thus of great importance to further investigate molecular mechanisms involved in pathological processes of KD, in order to identify new drug targets for a disease that is otherwise incurable and usually fatal.

Because of the lack of a curative treatment, management of KD is directed towards symptomatic relief. To date, stem cell transplantation is the only clinically available treatment, with limited evidence suggesting improved outcomes in pre-symptomatic patients. Recent success with fingolimod and siponimod as a treatment for relapsing-remitting and secondary progressive Multiple Sclerosis, respectively, has incited research to further investigate this compound as a potential candidate for therapeutic treatment of other neurodegenerative disorders. Promising findings from pre-clinical studies as well as clinical trials with both compounds justifies the need for further exploration into the effects of fingolimod and siponimod as potential pharmacological agents in neurodegenerative diseases, such as KD. In light of recent studies showing promising effects of both compounds on myelination and inflammation in organotypic slice cultures, the current research investigates the efficacy of those same drugs *in vivo* in twitcher mice, a murine model of KD. Findings from such preclinical studies will provide detailed information on toxicity and efficacy, thus setting the path towards clinical research trials. Additionally, using compounds that differentially target S1P receptors will inform about the relative importance of S1PR subtypes. Administration of siponimod as a selective S1PR_{1/5} agonist, compared to the unselective fingolimod, will provide more in depth information regarding receptors involved in remyelination and inflammation within the context of a neurodegenerative and demyelinating disease such as KD.

Outputs

Manuscripts

- **Béchet S**, Sullivan S, Yssel J, Fagan SG and Dev KK (2020). "Fingolimod rescues demyelination in a mouse model of Krabbe's disease." *The Journal of Neuroscience*: 2346-2319.
- **Béchet S** and Dev KK (2020), "Early administration of fingolimod reduces myelin debris, microglia reactivity and neuronal damage in twitcher mouse." SUBMITTED

Presented posters

- *Frontiers in Neurology (7th meeting), Dublin, Ireland*: Sphingosine 1-phosphate receptor modulation attenuates demyelination and inflammation in a murine model of Krabbe's disease. **Béchet S.**, Fagan S., O'Sullivan S., Yssel J., Dev K. (2017).
- *Glia, Edinburgh, UK*: Sphingosine 1-phosphate receptor modulation reduced demyelination in a murine model of Krabbe disease. **Béchet S.**, Fagan S., O'Sullivan S., Yssel J., Dev K. (2017).
- *Neuroscience Ireland, Galway, Ireland*: Sphingosine 1-phosphate receptor activation attenuates demyelination in the Twitcher mouse. **Béchet S.**, Fagan S., O'Sullivan S., Yssel J., Dev K. (2017).
- *FENS, Berlin, Germany*: Targeting the sphingosine 1-phosphate axis in a murine model of Krabbe's disease reduces demyelination. **Béchet S.**, Fagan S., O'Sullivan S., Yssel J., Dev K. (2018).
- *Royal Academy of Medicine in Ireland (RAMI), Dublin, Ireland*: The effect of FTY720 (fingolimod) on sphingosine 1-phosphate receptors in cerebellar slices of twitcher mice – a pre-clinical study. **Béchet S.**, Fagan S., O'Sullivan S., Yssel J., Dev K. (2018).
- *BNA, Dublin, Ireland*: The Sphingosine 1-phosphate axis as a promising target in a murine model of Krabbe's disease: Implications on inflammation and myelination. **Béchet S.**, O'Sullivan S., Fagan S., Dev K. (2019).
- *Glia, Porto, Portugal*: Targeting the sphingosine 1-phosphate axis leads to phenotypic improvements and reduced neuropathology in a murine model of Krabbe's disease. **Béchet S.**, O'Sullivan S., Fagan S., Dev K. (2019).

Scholarships and Awards

- Award for Best Project Prize 2016 within the M.Sc. Neuroscience, Trinity College Dublin.
- 3rd prize in the AMBER image competition (2016) of the Science Gallery, Trinity College Dublin, for the fluorescent image “stop twitching – start s’mylein” generated using confocal microscopy.
- Prize for best confocal image – Physiology Friday 2017.
- Awarded the TCD postgraduate studentship award (2018), covering registration fees and an annual maintenance of €6,500 for three years.
- Best Poster Prize – Neuroscience Ireland 2017, Galway, Ireland.
- Travel Awards from The Physiological Society, Neuroscience Ireland and Trinity College Dublin (2016-2019).
- Prize for best oral presentation – Physiology Friday 2019.

Chapter 1 – Introduction

1. Krabbe's Disease – A Lysosomal Storage Disorder

1.1. The spectrum of Krabbe's Disease: Epidemiology, Clinical Manifestations and Diagnosis

Globoid cell leukodystrophy (Krabbe's Disease; KD) is a rare lysosomal storage disorder with an autosomal recessive inheritance pattern affecting 1/100,000 live births in the United States and Northern Europe (**Wenger et al. 2000**). Since its first description in 1916 (**Krabbe 1916**), research has rapidly contributed to our molecular understanding of the KD neuropathology. Nevertheless, clinical practice still lacks a curative treatment, and management is mainly focused on symptomatic relief. The clinical phenotype is classified as either infantile or late-onset, based on the age at onset of manifestation (**Wenger et al. 2016**). KD predominantly affects infants, with the infantile form accounting for 85-90% of the cases (**Wenger et al. 2016**). However, less frequent juvenile and adult onset cases have been reported (**Debs et al. 2013, Wenger et al. 2016**). A later onset is thereby usually accompanied with a milder and heterogeneous clinical phenotype (**Debs et al. 2013, Wenger et al. 2016**).

Infantile KD typically develops within the first 6 months postnatal with rapid neurologic deterioration ensuring death within the first two years of age (**Graziano and Cardile 2015**). Hagberg and colleagues (**1969**) distinguished three stages of progression of the infantile KD that are still applicable today (**Table 1.1**). Hallmark symptoms of the classic infantile form include irritability, hypertonic spasticity and psychomotor stagnation, followed by rapid developmental decline, seizures and optic atrophy (**Graziano and Cardile 2015**). Clinical manifestations are thus translating the involvement of both first and second motor neurons, indicative of a systemic disorder affecting the central as well as the peripheral nervous systems.

Because of the rapidly progressing and invariably fatal nature of infantile KD, early diagnosis has become crucial for prognosis. To date, pre-natal diagnosis of at-risk-infants includes a combination of enzymatic and genetic assays (**Wenger et al. 2016**). Furthermore, since 2006 the United States have implemented a newborn screening for KD using dried blood spots to measure enzymatic activity with tandem mass spectroscopy (**Duffner et al. 2009, Orsini et al. 2016**).

Clinical features of later onset KD generally include a more gradual psychomotor regression, accompanied by progressive spastic paraparesis and gait abnormalities (**Shao et al. 2016**).

1.2. Galactosylceramidase: gene, protein and mutations

In 1970, Suzuki and Suzuki confirmed the deficiency of the lysosomal enzyme galactosylceramidase (GALC, EC 3.2.1.46) as source of the pathology in KD patients, thus classifying KD as a lysosomal storage disorder (**Suzuki and Suzuki 1970, Won et al. 2016**). The causative gene, *galc*, was consequently mapped to chromosome 14, encoding the precursor protein (80kDa) of 669 amino acid residues (**Zlotogora et al. 1990**). The precursor form is then processed to its mature form by cleavage to 50-kDa N-terminal fragment and 30-kDa C-terminal fragment in lysosomes (**Nagano et al. 1998, Won et al. 2016**). GALC is produced and glycosylated in the endoplasmic reticulum and then trafficked to the lysosome via the mannose-6-phosphate (M6P) pathway (**Nagano et al. 1998**). Structurally, GALC possesses three domains: a central triosephosphate isomerase (TIM) barrel, a β -sandwich domain, and a lectin domain (**Figure 1.1**) (**Deane et al. 2011**). The enzyme hydrolyses galactose ester bonds and is thus essential for normal catabolism of several glycosphingolipids (**Deane et al. 2011**). This is of particular importance for the metabolism of the principal lipid component of myelin galactosylceramide and the by-product of myelin turnover galactosylsphingosine (psychosine).

Various disease-causing mutations have been identified and reported to cause KD by deletions, frameshifts and missense mutations in *GALC* gene (**Graziano and Cardile 2015, Won et al. 2016**). Although the mutational profile is highly heterogeneous, the most common mutation affecting patients of European ancestry includes a 30-kb deletion that encodes non-functional protein (**Wenger et al. 2000**). Other mutations that may affect splicing, mRNA stability, or M6P transport pathway, as well as misfolding and premature degradation have been described throughout the three domains of the enzyme (**Deane et al. 2011**). In addition to GALC, galactosylceramide degradation requires the action of a nonenzymatic sphingolipid activator protein known as saposin A. This glycoprotein is essential for normal lysosomal degradation, and its genetic deficiency may thus contribute to KD pathology (**Spiegel et al. 2005, Deane et al. 2011**).

2. Mechanisms of Neuropathogenesis in Krabbe's Disease

2.1. Psychosine and galactosylceramide in Krabbe's Disease

Deficiencies of the lysosomal hydrolase GALC diminishes the ability of myelinating cells to break down glycolipids such as galactosylceramide and psychosine during myelin turnover, leading to their progressive accumulation. As a by-product of sphingolipid metabolism, psychosine is

synthesised by galactosylation of sphingosine by UDP-galactose ceramide galactosyl transferase (CGT) or by deacylation of galactosylceramide by N-deacylase (**Cleland and Kennedy 1960, Kanazawa et al. 2000**) (**Figure 1.2**). While barely present under normal conditions, cellular and tissue levels of psychosine have been shown to rise up to 100-fold under GALC-deficient conditions (**Suzuki 2003, Borda et al. 2008**). Unlike psychosine, galactosylceramide does not reach unusual concentrations in GALC-deficient cells and tissues. This major constituent of healthy myelin is generated by galactosylation of ceramide by UDP-galactose CGT (**Borda et al. 2008, Won et al. 2016**) (**Figure 1.2**). While it is a primary substrate of GALC, galactosylceramide can alternatively be hydrolysed by GM1 ganglioside β -galactosidase. Such compensatory actions allow maintaining reasonable levels of galactosylceramide in the brain of KD patients (**Borda et al. 2008**).

The leading hypothesis explaining the pathogenesis of KD is thus based on the loss of oligodendrocytes due to psychosine-induced cytotoxicity. The aggregation of psychosine is especially apparent in the white matter of the brain and in sciatic nerves, where psychosine may inhibit some critical cell processes which results in oligodendrocyte and Schwann cell apoptosis (**Zaka and Wenger 2004, Giri et al. 2008, Misslin et al. 2017**). This “psychosine hypothesis” was first formulated by Miyatake and Suzuki (**1972**), and has been endorsed by several *in vivo* and *in vitro* studies showing psychosine-induced cytotoxic effects (**Haq et al. 2003, Zaka and Wenger 2004, Giri et al. 2006, Giri et al. 2008**). Nevertheless, the exact mechanism of action by which psychosine exerts cytotoxic effects remains to be fully elucidated.

2.2. Mechanisms underlying psychosine toxicity

While the enzymatic synthesis of psychosine has been more or less established, its mechanisms of toxicity remain unclear. Given the broad spectrum of psychosine-mediated repercussions, it seems unlikely that its toxicity is mediated through a single signalling pathway. Attempts at understanding psychosine-induced apoptosis have uncovered various mechanisms of actions, involving multiple cell signalling pathways. It has been suggested that cytotoxic effects act via mitochondrial dysfunctions, caspase activation, peroxisomal dysfunctions, accumulation of lipid rafts and alteration of the protein kinase C signalling, and NF κ B pathways (**Haq et al. 2003, Zaka and Wenger 2004, Haq et al. 2006, White et al. 2009**). More recently, psychosine has been found to play a role in α -synuclein fibrillisation, creating neuronal inclusions of aggregated misfolded protein that contribute to cell death (**Smith et al. 2014**).

- **Psychosine-induced apoptosis and oxidative stress**

Exogenous psychosine has been shown to activate various pro-apoptotic signalling pathways involving caspase 3/9 activation and up-regulation of JNK (**Haq et al. 2003, Zaka and Wenger 2004**). Moreover, psychosine was shown to induce alterations of mitochondrial redox, such as reduced action potential, cytochrome C release and generation of reactive oxygen species (ROS) (**Haq et al. 2003, Voccoli et al. 2014**). Such pro-apoptotic signalling seems to be accompanied by inhibited cell survival, such as Akt and Erk signalling cascades (**Haq et al. 2003, Won et al. 2013**). In addition to apoptosis, psychosine increases levels of oxidative stress, as seen in cultured cells treated with exogenous psychosine (**Haq et al. 2003, Giri et al. 2008**).

- **Psychosine involvement in secretory Phospholipase A₂ cytotoxicity**

Secretory Phospholipase A₂ (sPLA₂) is part of a family of enzymes involved in the hydrolysis of glycerophospholipids. Under pathological conditions, sPLA₂ activation has been shown to cause inflammation, mitochondrial depolarisation, and oxidative stress (**Won et al. 2016**). Studies with twitcher mice and *in vitro* studies using psychosine-treated oligodendrocytes and astrocytes support the detrimental role of sPLA₂ signalling in psychosine-mediated oxidative stress and cell apoptosis (**Haq et al. 2003, Giri et al. 2006, Misslin et al. 2017**).

- **Psychosine triggers peroxisomal dysfunctions**

Peroxisomes are subcellular organelles that play an essential role in various cellular functions, including the lipid metabolism, oligodendrocyte differentiation and myelin synthesis (**Kassmann 2014**). Their importance in myelin metabolism is particularly emphasised in peroxisomal disorders, such as Zellweger spectrum or X-linked adrenoleukodystrophy, where mutations of peroxisomal proteins lead to severe white matter abnormalities (**Kassmann 2014**). Similarly, psychosine has been shown to disrupt the cellular homeostasis of peroxisomes, by inhibiting peroxisome proliferator-activated receptors (PPARs) via activation of sPLA₂ pathway (**Haq et al. 2006, Singh et al. 2009**).

- **Psychosine-induced disruption of the lipid raft architecture**

Mechanistic studies of psychosine mediated toxicity have shown that psychosine accumulates preferentially in cell membrane microdomains, termed lipid rafts (**White**

et al. 2009, White et al. 2011). Lipid rafts are sphingolipid- and cholesterol-rich areas of membrane architecture and facilitate processes of cellular signalling (**White et al. 2009**). Their disruption has been suggested to play a compounding role in binding or activation/inactivation of kinases and phosphatases, particularly protein kinase C (PKC), an important signal transduction protein (**White et al. 2011, Hawkins-Salsbury et al. 2013**). Psychosine has been shown to inhibit the translocation of PKC to the plasma membrane (**White et al. 2011, Hawkins-Salsbury et al. 2013**). PKC is involved in many different signalling cascades and regulates myelin gene expression and process formation within oligodendrocytes. Neurotoxicity caused by faulty interaction of raft lipids with cytoskeletal proteins is thus an important focus for research efforts.

- **Axonal pathology and synaptic failure: Neuropathy, axonopathy and neuromuscular dysfunctions**

Severe demyelination in the PNS has been regarded as the main cause for KD-characteristic skeletal muscle atrophy. However, growing evidence suggests that myelin-independent neuronal injuries may exacerbate the KD neuropathology. Axonal and neuronal degeneration has been reported in human KD patients and in twitcher mice, where psychosine was shown to accumulate in both glia and neurons (**Castelvetri et al. 2011, Cantuti-Castelvetri et al. 2015**).

2.3. Beyond the psychosine hypothesis: novel explanatory approaches

While the “psychosine hypothesis” has been the predominant explanation for KD pathology for over 40 years, a study using a different causative mutation in *twi-5J* has found disparities between psychosine accumulation and demyelination in the CNS (**Potter et al. 2013**). Such findings challenge the longstanding hypothesis attributing the underlying pathophysiological progression of KD solely to psychosine aggregation. Instead, the early demise may be partly ascribed to neuroinflammation rather than myelin loss (**Potter et al. 2013**). A study by Kondo and colleagues (**2005**) showed that GALC-deficient oligodendrocytes from twitcher mice were able to myelinate axons in the shiverer mouse upon transplantation. This suggests that the severe demyelination in KD is not exclusively imputable to oligodendrocytes dying by lipid accumulation, but may be catalysed by inflammatory processes, rather than psychosine alone. Furthermore, recent studies examining early pathological changes in KD have demonstrated that changes in or loss of myelin is preceded by neuroinflammation, with reactive microglia forerunning astrogliosis (**Potter et al. 2013, Snook et al. 2014**). Such emerging evidence seems

in favour of a “microglial hypothesis”, postulating that inflammatory processes precede oligodendrocyte death and that demyelination occurs as a consequence of activated astrocytes, microglia and globoid cell formation **(Nicaise et al. 2016)**.

2.4. Immune and inflammatory mechanisms in Krabbe’s Disease

Leukodystrophies, such as KD, are commonly associated with severe white matter degeneration as their most prominent feature. However, activation of the innate immune response generally accompanies disease progression and thus constitutes a defining component of the KD pathology. While psychosine-mediated cytotoxicity to myelinating cells has been established, its role in immune activation needs to be further elucidated. Several research groups have reported early pro-inflammatory cytokine and chemokine expression in presymptomatic twitcher mice, with further increases with disease progression **(Haq et al. 2003, Haq et al. 2006)**. Exogenous psychosine at concentrations of 10 μ M or higher has been shown to affect apoptosis, sphingosine 1-phosphate signalling, peroxisomal and mitochondrial functions, PKC, TNF α and iNOS among others **(LeVine et al. 2000, Haq et al. 2003, Giri et al. 2006)**. Psychosine mediated inflammation could, in turn, further contribute to oligodendrocyte and astrocyte apoptosis **(Giri et al. 2006)**. Cell death induced by pro-inflammatory cytokines has been shown to play an additional exacerbating role in KD, in addition to direct psychosine-mediated toxicity **(Giri et al. 2006, Haq et al. 2006)**. However, exogenous psychosine is likely to generate different cellular responses than endogenous accumulation. Data from such *in vitro* studies thus need to be interpreted with care and supported with further *in vivo* studies. In addition to direct actions of psychosine, neuropathology in KD and twitcher mice is thought to be partially driven by phagocytic microglia. Myelin and oligodendrocyte debris occurring during demyelination activate microglial cells, which, in turn, mediate inflammatory response **(Avola et al. 2016)**. Those phagocytic cells secrete a variety of cytokines that augment and perpetuate inflammatory events in KD. CNS inflammation during progressive accumulation is thus a prominent histopathologic feature of KD. A particular feature and histological hallmark of KD is the presence of multinucleated macrophages termed ‘globoid cells’, that complement the classic neuroinflammatory components **(Suzuki and Suzuki 1970)**. Although the origins of those phagocytic cells remain unclear, they are thought to originate from CNS-resident microglia and/or infiltrating peripheral macrophages. This has been based on immunopositivity of globoid cells to ferrin, CD68 and Iba1, all of which are markers for microglia/macrophages **(Nicaise et al. 2016)**. Regardless of their origin, globoid cells are thought to result from demyelination and inflammation. They usually contain Periodic-Acid Schiff(PAS)-positive debris, suggesting high

levels of phagocytic activity of galactosylceramide, a cerebroside native to the myelin sheath. However, recent evidence attributes an earlier pathological role to those phagocytic cells, showing that the presence of globoid cells precedes demyelination (**Potter et al. 2013, Potter and Petryniak 2016**). Together, studies investigating immune mechanisms in KD give robust evidence suggesting that the loss of oligodendrocytes and the concurrent demyelination characteristic for KD are due to psychosine induced apoptotic processes as well as aberrant inflammatory responses.

Stage	Major Clinical Phenotypes
Stage I	General irritability; hypertonic spasticity auditory, tactile and visual hyperesthesia. Retardation or regression of motor and mental development. Feeding impairment; gastroesophageal reflux with vomiting; progressive weight loss.
Stage II	Severe motor and mental deterioration. Hypertonicity; back arching, myoclonic seizures. Optic atrophy and visual loss.
Stage III	Decerebrate posture; absence of voluntary movements; hypotonia. Blindness; respiratory infections; hyperpyrexia; deafness.

Table 1.1 Stages of Progression of Infantile Krabbe's Disease

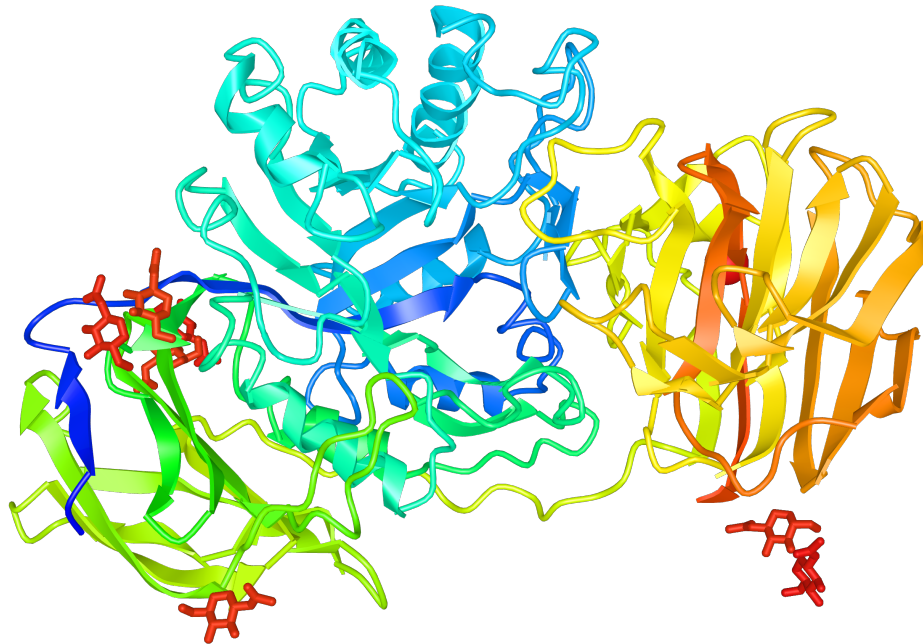


Figure 1.1 Structure of murine GALC

Ribbon diagram of mouse GALC coloured by domain: beta-sandwich (yellow), TIM barrel (blue), lectin domain (green). The murine protein has been shown to have 82% identity with human GALC (**Deane et al. 2011**).

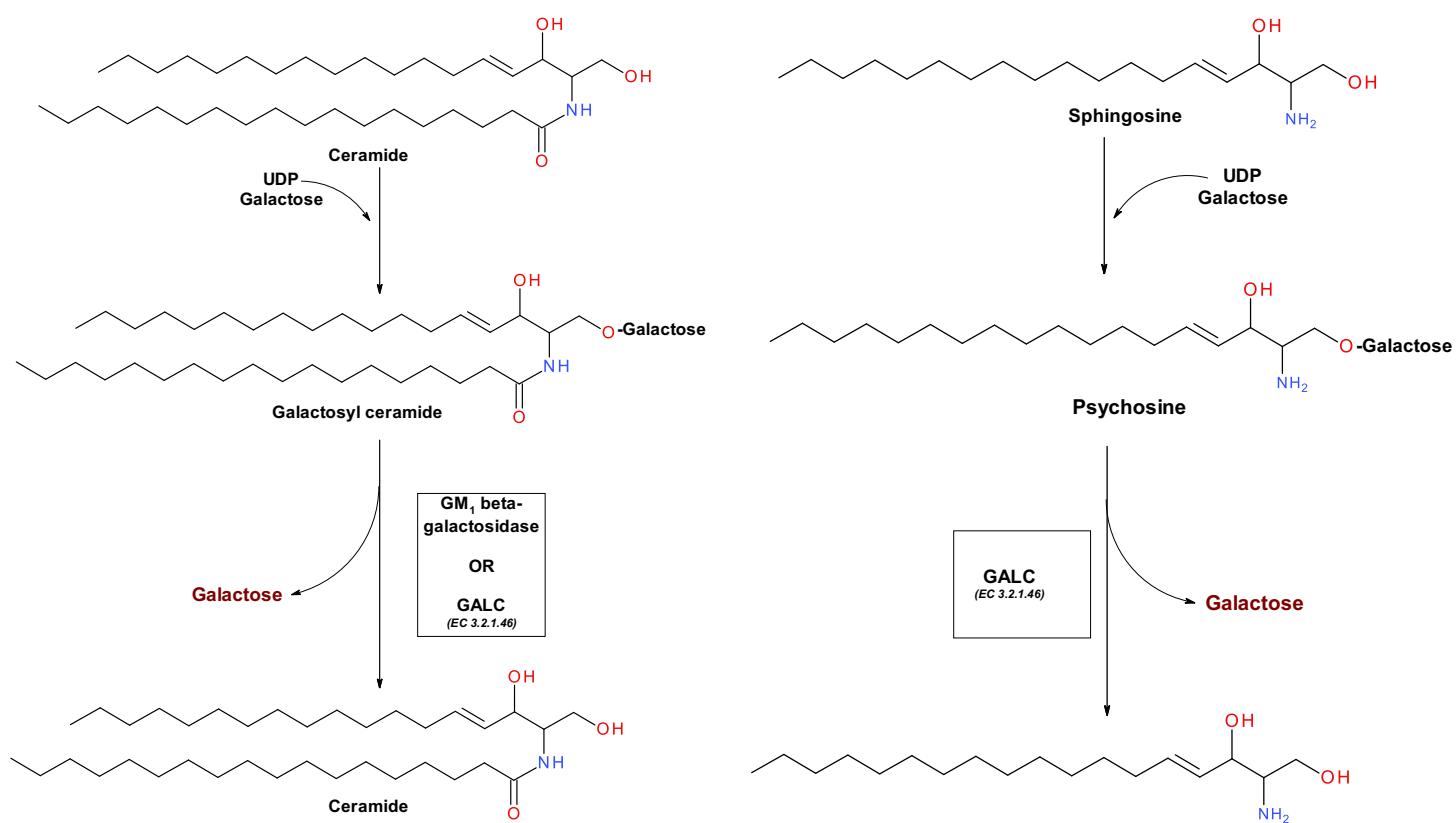


Figure 1.2 Catabolic Pathways of psychosine and galactosylceramide

Figure drawn with BIOVIA Draw software.

3. Animal Models of Krabbe's Disease

3.1. The Twitcher mouse model (B6.CE-Galc^{twi}/J)

The twitcher mouse was first observed in 1976 and maintained on a mixed C57BL/6J and CE/J background at the Jackson Laboratory (**Duchen et al. 1980**). Caused by spontaneously arising mutations in the gene coded for galactosylceramidase, the twitcher mouse constitutes an enzymatically authentic model of humane KD. While indistinguishable from normal littermates at birth, homozygous mutation carriers start to show clinical manifestations characteristic for KD at approximately 20 days postnatal. Subsequent disease progression is rapid with mice rarely surviving beyond PND37-40 (**Duchen et al. 1980, Suzuki 1995**). The most prominent features of the murine model include muscle weakness in the hindlimbs with progressive weight loss as well as the eponymous twitching and tremors. Contrary to humans, however, twitcher mice do not exhibit seizures or decerebrate rigidity (**Suzuki 1995**). Histologically, the disease mostly aligns with human KD findings, with psychosine-induced toxicity as a primary cause for pathogenesis (**Suzuki, 1995**). The twitcher CNS is characterised by severe demyelination, along with astrogliosis. Furthermore, both central and peripheral nervous systems contain multinucleated globoid cells (**Suzuki, 1995**). However, differences in demyelination onset and extent have been observed (**Kobayashi et al. 1986**). Such differences in disease phenotype between murine and human KD, as well as the small size and limited life span of the animal reduce the translational utility of this model.

Nevertheless, owing to a high degree of genetic manipulability, low cost of breeding and generation interval, the twitcher mouse model counts among the most frequently used and invaluable models for investigation of KD neuropathology. Further use of this model is observed in KD therapeutics when testing the effectiveness of bone marrow transplantation, viral vector gene or enzyme replacement therapies.

3.2. The Twitcher 5 Jackson model (BXD32/TyJ-Galc^{twi-5J}/J)

The Galc^{twi-5J} mouse carries a spontaneous mutation in GALC that matches the missense mutation in patients with infantile KD. It homozygotes on the BXD32/TyJ background and its pathology is characterised by an earlier onset than the original Twitcher mutation on a B6.CE background (**Karst et al. 2008**). Moderate tremors can already be detected between PND14-16, accompanied by rapid weight loss, smaller body size and an average life span of 21 days (**Karst et al. 2008, Potter et al. 2013**). Pathologically, animals exhibit the main neuropathological

hallmarks including globoid cell infiltration and psychosine accumulation (**Potter et al. 2013**). No direct correlation between psychosine accumulation and myelin loss was observed, indicating that psychosine accumulation in this model may not directly lead to demyelination but rather due to ongoing exacerbating inflammatory processes (**Potter et al. 2013**). Finally, the PNS manifests a profound hypomyelination, associated with abnormal myelination and axonal loss of peripheral nerves at all ages (**Potter et al. 2013**). This suggests that myelination in this model is deficient rather than lost due to degeneration (**Karst et al. 2008**).

3.3. The Rhesus Macaque Model

The rhesus monkey model is the first nonhuman primate species that has been reported to develop a lysosomal storage disorder. The disease-causing mutation can be traced back to a deletion in exon 4 and the deduced amino acid sequence of the monkey GALC enzyme was found to be 97% identical to human GALC (**Luzi et al. 1997, Borda et al. 2008**). Life expectancy of affected monkeys does not exceed 5-6 months and low lysosomal enzyme activity and subsequent psychosine accumulation results in KD-like symptoms including tremors, hypertonia, and ataxia (**Baskin et al. 1998**). Pathologically, cerebral, cerebellar and spinal cord white matter show severe demyelination accompanied by infiltration of multinucleated globoid cells (**Baskin et al. 1998**). The PNS is characterised by decreased numbers of peripheral nerve fibres and reduced myelin as well as marked decreases in nerve conduction velocity (**Weimer et al. 2005, Borda et al. 2008**). A particular benefit associated with the use of the rhesus model in the study of KD is the high degree of similarity between human and macaque CNS. This is especially useful for exploring treatment options such as prenatal bone marrow transplantation and different approaches to gene therapy.

3.4. The Canine Model

Since its first description in 1963 in cairn and West Highland white terriers, the canine model has been asserted as a reliable animal model for research on KD pathology. The mutation in the *GALC* gene resulting in a non-functional mutant protein compares to the nonsense mutation of the twitcher mouse and the frameshift mutation of the rhesus monkey (**Wenger et al. 1999, Bradbury et al. 2016**). MRI findings of affected dogs align with those of human patients, thus providing a promising way to test therapeutic effectiveness (**Wenger et al. 1999**). Clinically, KD symptoms appear between the first and third month of age with an overall life expectancy of 8

to 9 months (**Wenger et al. 1999, Cantuti-Castelvetri et al. 2015**). The naturally occurring KD mutation in the canine model mirrors the pathological and biochemical abnormalities of human disease (**Cantuti-Castelvetri et al. 2015, Bradbury et al. 2016**). Additionally, the similarities between canine and infant brains allows for the evaluation of various therapies using identical diagnostic, imaging and biomarker techniques to those used in children (**Bradbury et al. 2016**). The canine model this provides an intermediate step in advancing therapies from the murine model into clinical practice.

4. Experimental therapies for Krabbe's Disease

The current standard care for KD is hematopoietic stem cell transplantation (HSCT), derived from bone marrow or umbilical cord blood. The introduction of donor-derived GALC positive cells leads to their migration into the CNS where they provide GALC activity. GALC secreted by donor cells can then be taken up by deficient cells in the CNS (**Allewelt et al. 2018**). Additionally, recent studies have shown that HSCT provides immunomodulatory functions, although its mechanism of action remains to be elucidated (**Reddy et al. 2012**). It is thus likely that HSCT therapeutic effects can be traced back to both supplied GALC activity as well as reduced neuroinflammation. Several studies using twitcher mice have shown increased GALC activity with improved myelination and increased lifespan (**Kondo et al. 2005, Reddy et al. 2012**). However, while treatment of juvenile and adult patients has been more promising, HSCT outcomes remain modest in infantile patients. If initiated during early stages of disease, HSCT has been shown to slow the disease progression. However, its failure to correct peripheral neuropathy in infants is one of the main drawbacks of HSCT (**Wenger et al. 2016**). If initiated *in utero*, HSCT has proven unsuccessful ensuring death during the third trimester or shortly after birth (**Bambach et al. 1997**).

Enzyme replacement therapy (ERT) has shown promise in several other lysosomal storage diseases, thus warranting further research into restoring functional GALC activity in KD. Studies have shown that single intraperitoneal or intracerebroventricular (i.c.v) administration of recombinant GALC were able to significantly increase lifespan of twitcher mice (**Lee et al. 2005, Lee et al. 2007**). Direct CNS injections via i.c.v. also reduced psychosine accumulation (**Lee et al. 2007**). However, the incapacity of GALC to cross the blood brain barrier would require multiple dosing sites throughout both CNS and PNS in order to counteract KD pathology. While pre-clinical studies suggest that repeated administration would likely result in improved therapeutic efficacy of ERT, such invasive procedures are unlikely to be adopted in clinical practice.

With only partially successful results from BMT and ERT, research has expanded to investigate the efficacy of CNS directed gene therapy. Studies using murine and canine models of KD have shown that adeno-associated viral (AAV)-mediated gene therapy increased enzymatic activity in both CNS and PNS. Additionally, it improved lifespan as well as clinical features of those animals (**Rafi et al. 2015, Bradbury et al. 2018**). These positive outcomes seem to be enhanced in the twitcher mouse when AAV-mediated gene therapy is administered in combination with BMT (**Rafi et al. 2015**).

Therapies such as substrate reduction, antioxidant or anti-inflammatory therapies have revealed promising in ameliorating KD phenotypes and extending lifespan. While such therapies fail to directly impact enzymatic deficiency, they provide tools to slow down disease progression. Substrate reduction with L-cycloserine, an inhibitor of 3-ketodihydrosphingosine synthase, extended the lifespan and decreased astrogliosis in twitcher mice (**LeVine et al. 2000**). Antioxidant therapy through high-vitamin-D diet resulted in a slower rate of psychosine accumulation and reduced levels of demyelination in twitcher mice (**Paintlia et al. 2015**). Beside strong demyelination and oligodendrocyte death, KD is characterized by severe neuroinflammation. Several anti-inflammatory compounds such as ibudilast, indomethacin, ibuprofen and micocycline have been shown to reduce levels of inflammatory cytokines and increase lifespan in murine models of KD (**Kagitani-Shimono et al. 2005, Luzi et al. 2009**).

While some experimental studies have resulted in a significant extension of lifespan as well as improved myelination status, translation into clinical practice remains impossible due to ethical constraints. To date, no treatment or experimental therapy has resulted in a complete correction of the clinical and pathological features associated with KD and management is mainly focused on symptomatic relief, physical therapy and nutritional support.

5. Sphingosine 1-Phosphate Receptors in Health and Disease

5.1.Sphingosine 1-Phosphate: Metabolism and Mechanism of Action

Sphingosine 1-Phosphate (S1P) is a membrane-derived lysophospholipid that has emerged as a central regulator of many vital cellular processes, including cellular proliferation and survival, migration, differentiation as well as the control of lymphocyte trafficking, angiogenesis and vascular integrity (**Brinkmann et al. 2004**). S1P is predominantly blood borne with sources such as thrombocytes, erythrocytes and endothelial cells. As a small lipid in extracellular space, S1P is bound by plasma and serum proteins which provide a stable supply and efficient delivery to most tissues (**Brinkmann et al. 2004, Brinkmann 2007**). Physiological levels of S1P in various

tissues are predominantly maintained by the balanced activity of two sphingosine kinases, namely SphK1 and SphK2, along with S1P phosphatases and S1P lyase (**Figure 1.3**) (**Le Stunff et al. 2002, Pappu et al. 2007**). Following its intracellular production, S1P is transported out of cells where it acts on a family of five G protein-coupled receptors (GPCRs), termed S1PR₁₋₅, previously known as members of the endothelial differentiation gene (EDG) family (**Brinkmann 2007**). Those receptors are widely expressed throughout the body, and bind to S1P with high affinity (**Mendelson et al. 2014**). Although S1P predominantly acts as an extracellular signalling molecule, it may also function as an intracellular messenger through HDAC and TRAF2 before being broken down by S1P lyase (**Alvarez et al. 2010, Fyrst and Saba 2010**). Moreover, S1P has also been shown to act through GPCR-independent pathways, such as prohibitin 2 or TRAF2 (**Alvarez et al. 2010, Strub et al. 2011**).

5.2. Sphingosine 1-Phosphate Receptor Expression and Functions in the CNS

S1PRs are expressed ubiquitously and are invaluable in cell survival, growth and differentiation. They are involved in a range of unique biological functions, including immune cell trafficking, cardiovascular homeostasis and cell communication in the CNS (**Blaho and Hla 2014**). It is thus not surprising that S1PR family plays an important role in embryonic development with its absence leading to embryonic lethality in S1PR-null animal models (**Kono et al. 2004, Means et al. 2007**). While receptor subtypes S1PR₁₋₃ are generally known to be widespread throughout the body, including immune, cardiovascular and central nervous systems, S1PR₄ and S1PR₅ show more selective expression patterns. S1PR₄ mainly resides in lymphatic tissue and the location of S1PR₅ has mainly been narrowed to natural killer cells as well as oligodendrocytes in white matter tracts of the CNS (**Brinkmann 2007, Choi and Chun 2013**). The differential G-protein coupling of individual S1PR subtypes and their signalling pathways is summarised in **Figure 1.4**.

Four out of the five S1PR subtypes are expressed in the CNS, and include S1PR_{1-3,5}. All four receptors are expressed on neurons, astrocytes, microglia and oligodendrocytes with a varying level of expression depending on temporal, spatial and environmental conditions (**Figure 1.5**) (**Choi and Chun 2013**). In astrocytes, S1PR_{1,3} are dominant, with a lower expression of S1PR₂. S1PR₅ expression on astrocytes seems very low at basal levels, but is increased during growth factor exposure in culture (**Rao et al. 2004**). When exposed to pathogens, reactive astrocytes up-regulate S1PR_{1,3} simultaneously, suggesting synergistic or antagonistic functions on intracellular signalling cascades (**Choi and Chun 2013**). Upon activation, both receptors have been shown to be involved in astrocyte migration, proliferation and activation (**Sorensen et al.**

2003, Choi et al. 2011). Interestingly, S1P-mediated astrogliosis has been associated with both neuronal damage and repair (**Czech et al. 2009, Miron et al. 2010, Choi et al. 2011**). Such contradicting evidence seems to suggest that S1P-related astrogliosis may be partly dictated by the existence of contextual variables such as chronic versus acute disease conditions (**Choi and Chun 2013**). In microglia, relative levels of expression of S1PRs vary with their activation state. For instance, S1PR₂ dominates over S1PR₁ and S1PR₃ in activated microglia (**Choi and Chun 2013**). Furthermore, it has been shown that S1PR₁ deletion in CNS cell lineages can reduce microglial activation, making S1PR modulation on microglia a valuable target for drug development (**Choi et al., 2011**). Oligodendrocytes show a more restricted pattern of S1PR expression, mainly limited to S1PR₅ (**Jaillard et al. 2005, Snaidero and Simons 2014**). S1PR₅ plays an important role in cell survival of mature oligodendrocytes, and is consequently thought to constitute a vital component in myelination. *S1P5* knockout mice, however, seem to show no obvious myelination defects (**Jaillard et al. 2005**). Interestingly, *S1P1* conditional knockout mice lacking S1P₁ in oligodendrocyte lineage cells presented with a decreased expression of myelination proteins and reduced myelination in the corpus (**Kim et al. 2011**). Such findings attribute a substantial role to S1PR₁ in the formation of healthy myelin. In neurons, S1P signalling regulates a variety of functions, including migration, apoptosis and survival, synapse formation and synaptic transmission (**Dev et al. 2008**). Similar to glial cells, differential S1PR expression in neuronal cells can trigger antagonistic signalling pathways. For instance, S1PR₁ transactivation through nerve growth factor is associated with enhanced neurite extension, while S1P_{2,5} signalling inhibits this process (**Toman et al. 2004**).

5.3.S1P Receptor modulation through FTY720 (Fingolimod)

FTY720 (Gilenya®/fingolimod) constitutes a *bona fide* regulator of S1PRs and entered the clinical research pipeline for Multiple Sclerosis (MS) patients with the beginning of the first Phase II trial in 2003 (**Kappos et al. 2018**). Since then, several Phase II, Phase III and extension trials have shown beneficial results for a number of diseases. Fingolimod (Gilenya®) is currently approved for second line treatment of MS in Europe and FDA-approved for first-line use in the United States. Fingolimod is structurally close to endogenous S1P and is phosphorylated by SphK to its active form, fingolimod phosphate (pFTY720) (**Brinkmann 2007**). As a highly lipophilic drug, fingolimod can easily pass the blood-brain barrier and penetrate the CNS, where it is phosphorylated. Once phosphorylated to its active form, pFTY720 behaves as a full agonist on S1PR₁, S1PR₄ and S1PR₅ at low nanomolar concentrations, and as a partial agonist on S1P₃

receptors (**Dev et al. 2008**). It is thought to be inactive on S1PR₂, although this remains controversial (**Brinkmann 2007**). Fingolimod-mediated activation of S1PR₁ expressed on lymphocytes has been shown to down-regulate immune cell trafficking, by inducing receptor internalisation. As a consequence, S1PR mediated lymphocyte egress from the thymus and lymph nodes is attenuated resulting in prolonged and profound lymphopenia. The ensuing immunosuppressive outcome restricts T cells migration to sites of inflammation, thus prolonging allograft survival in preclinical models of transplantation (**Liu et al. 2015**). Most importantly, beneficial effects of fingolimod have been demonstrated in preclinical and clinical studies for treatment of relapsing-remitting MS and other neurodegenerative diseases (**Calabresi et al. 2014, Kappos et al. 2015**). In addition to its immunomodulatory functions, fingolimod is postulated to play a protective role via direct S1PR modulation on glial and neural cells within the CNS (**Miron et al. 2010, O'Sullivan and Dev 2015**).

5.4.S1PR modulation through BAF312 (Siponimod)

Since the development of fingolimod and its demonstrated clinical efficacy in MS, there have been ongoing efforts to develop more selective drug candidates targeting the S1P axis. These compounds are primarily directed towards selective agonism or antagonism of S1P receptor subtypes 1 and/or 3. Reasons for this are found in the mechanism of action of both receptors: S1PR₁ is strongly involved in the regulation of inflammatory responses, and S1PR_{1/5} have been postulated to promote myelination state (**Choi and Chun 2013**). S1PR₃ however has been shown to induce bradycardia (**Mazurais et al. 2002**). BAF312 (siponimod) is a selective S1PR_{1/5} dual agonist that has been developed by modifying the chemical structure of fingolimod, resulting in a shorter elimination half-life (**Pan et al. 2013, Biswal et al. 2015**). Pre-clinical studies in rodents show that siponimod is able to suppress clinical symptoms in EAE, without however avoiding bradyarrhythmic events (**Gergely et al. 2012**). Similar findings have been observed in healthy subjects, which can be mitigated by polypharmacy with propranol or by using dose-titration schemes (**Biswal et al. 2015**). Siponimod (Mayzent®) was developed by Novartis Pharmaceuticals, and was licensed by the European Medicines Agency in January 2020 for the treatment of secondary progressive MS (**Al-Salama 2019**). Currently, siponimod is being investigated in clinical trials of various phases in patients with hepatic impairment (NCT01565902), polymyositis (NCT01801917), dermatomyositis (NCT02029274) or haemorrhagic stroke (NCT03338998).

6. Targeting the S1P Axis in neurodegeneration and beyond

The past number of years have witnessed an increased interest in the therapeutic potential of S1PR modulation, with numerous clinical trials and pre-clinical research investigating S1P metabolism in a variety of diseases (**O'Sullivan and Dev 2017**). Compounds targeting S1P receptors are manifold, with the leading candidates including fingolimod and siponimod (**Table 1.2**). In addition to developing S1P receptor agonists/antagonists, research efforts are also turning towards targeting the S1P axis on other levels, for instance by inhibiting sphingosine kinases or S1P lyase activity, thus affecting S1P synthesis, degradation and export (**Kunkel et al. 2013**).

Pre-clinical studies are highlighting the importance and effectiveness of S1PRs in downregulating detrimental effects in many diseases, including neurodegenerative, neurological and psychiatric disorders. The majority of these studies thereby relies on the construction of both transgenic and gene knockout animal models, with both techniques providing complementary approaches to elucidating the role of S1P signalling. Specific gene editing using knockout procedures allows to mimic more subtle mutations. For example, using female sphingosine kinase-1 knockout mice, Gorshkova et al. (**2013**) found that S1P signalling was vital in animal resuscitation following cardiac arrest. Similarly, *S1P5* deficient mice were used to investigate the role of that receptor on oligodendroglial cell lineage proliferation and survival (**Jaillard et al. 2005**). Transgenic animal models on the other hand allow to study the function of specific genes and/or pharmacological compounds at the level of the whole organism. Fingolimod was found to improve motor function in a transgenic mouse model of Huntington's disease, and was shown to reduce inflammation in a murine model of traumatic brain injury (**Di Pardo et al. 2014, Mencl et al. 2014**). These emerging and promising results from *in vivo* and *in vitro* studies validate initiation of clinical trials investigating the efficacy, tolerability and safety of S1P axis modulation in several CNS related diseases including but not limited to ALS, epilepsy, Rett syndrome, glioblastoma and acute stroke (**Kunkel et al. 2013, O'Sullivan and Dev 2017**).

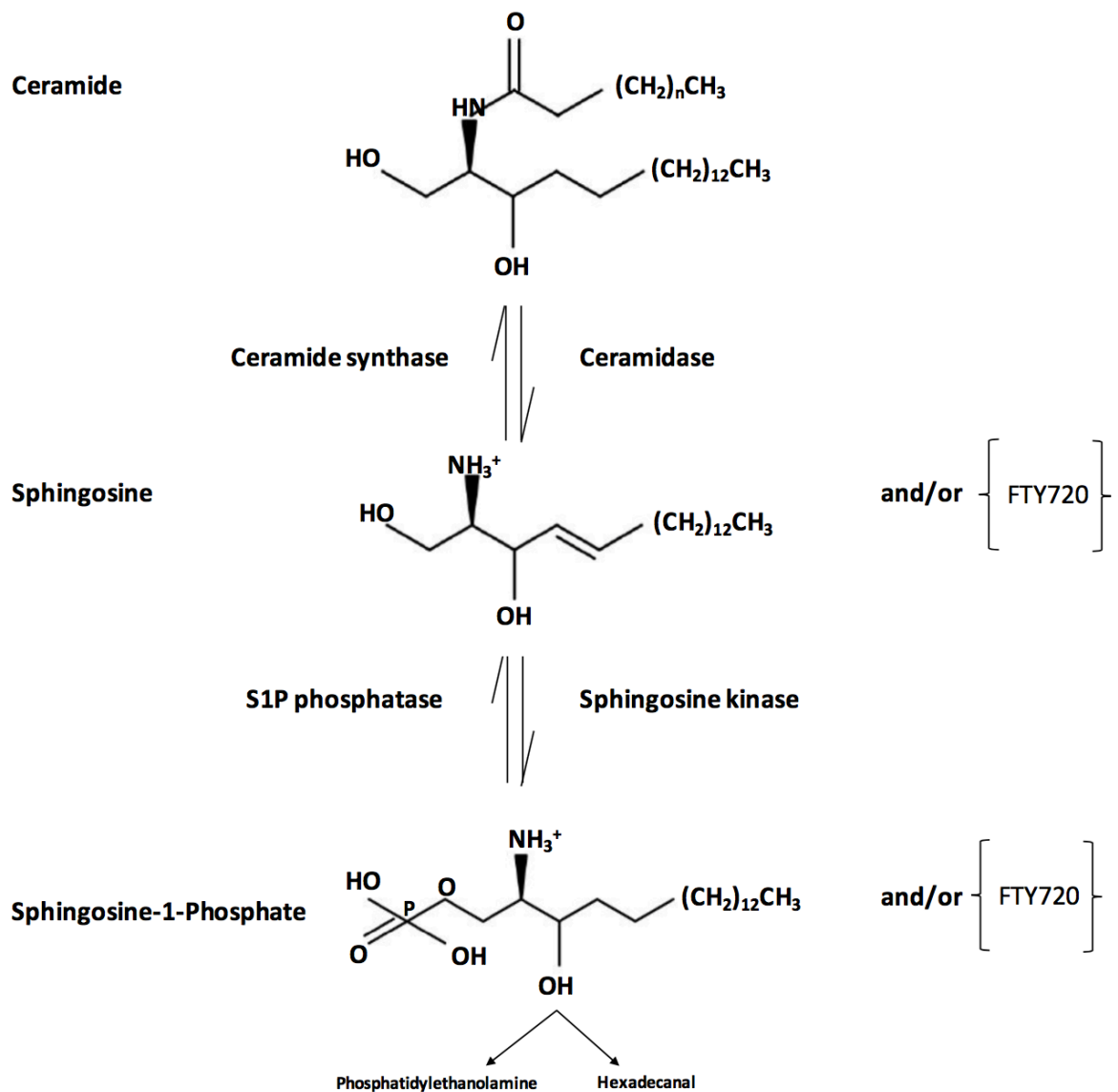


Figure 1.3 S1P metabolic pathway

S1P is formed from sphingosine by sphingosine kinases (Sphk) 1/2 and degraded by S1P lyase. Its precursor sphingosine can also be used to synthesise the cell-toxic metabolite psychosine. The reverse reaction, degradation of psychosine resulting in sphingosine, is catalysed by the enzyme GALC, which is defective in KD. S1P promotes cell growth and inhibits apoptosis, while its precursors, ceramide and sphingosine, typically inhibit cell growth and induce apoptosis. Therefore, the cellular balance of these three sphingolipid metabolites (sphingolipid rheostat) is of crucial importance in regulating cell fate.

S1P: Sphingosine-1 phosphate, R: Ethanolamine or choline.

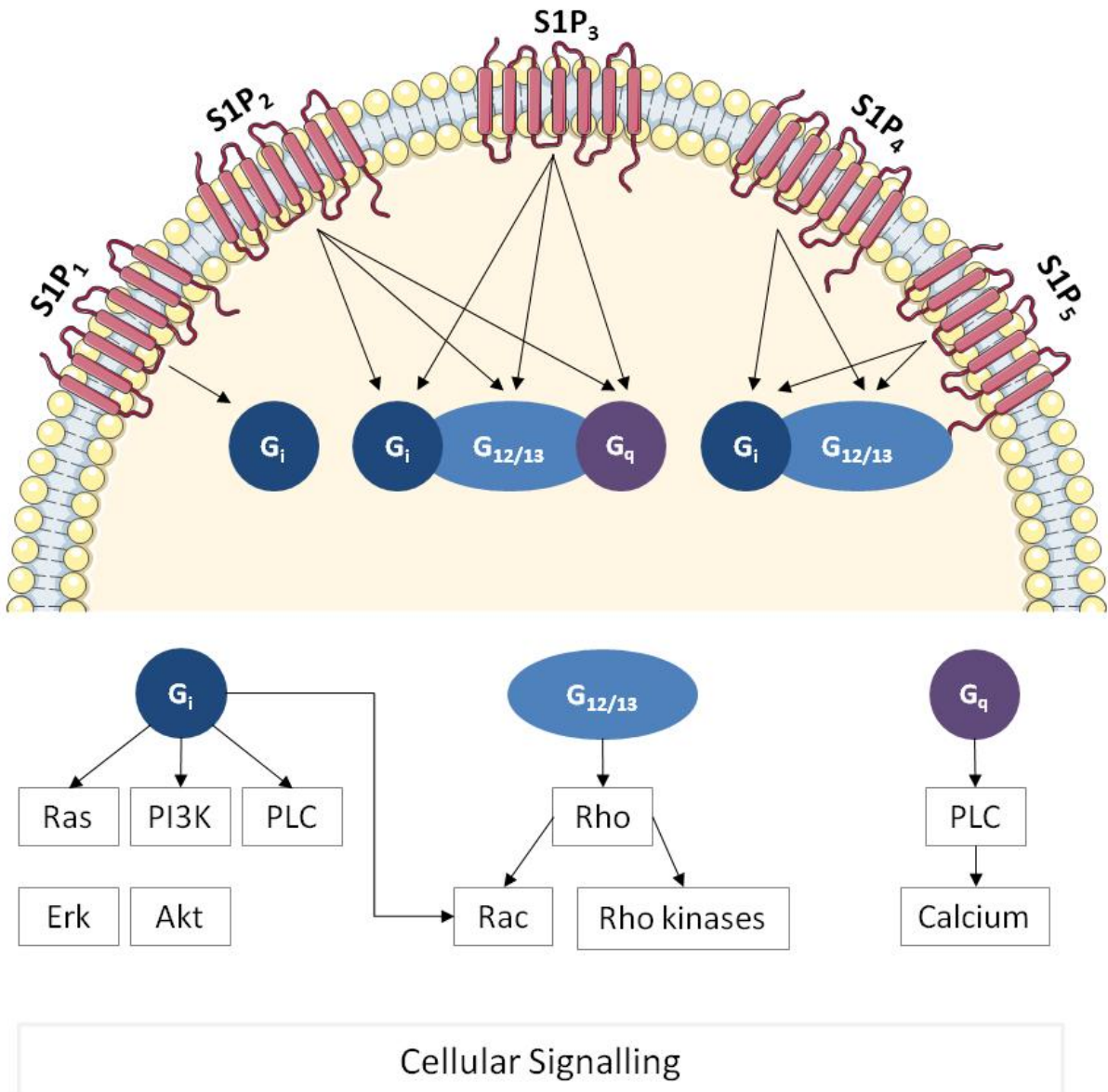


Figure 1.4 S1P receptors and their G-protein-coupled signalling pathways

Activation of S1PRs triggers several well characterised signalling pathways which, in turn, can promote a variety of cellular functions, including cell proliferation and survival, cell migration, vascular tone, endothelial barrier function and neural cell communication.

PI3K, Phosphoinositide 3-kinase; Ras, small GTPase; PLC, phospholipase C; Rho, small GTPase; ERK, extracellular signal-regulated kinase,

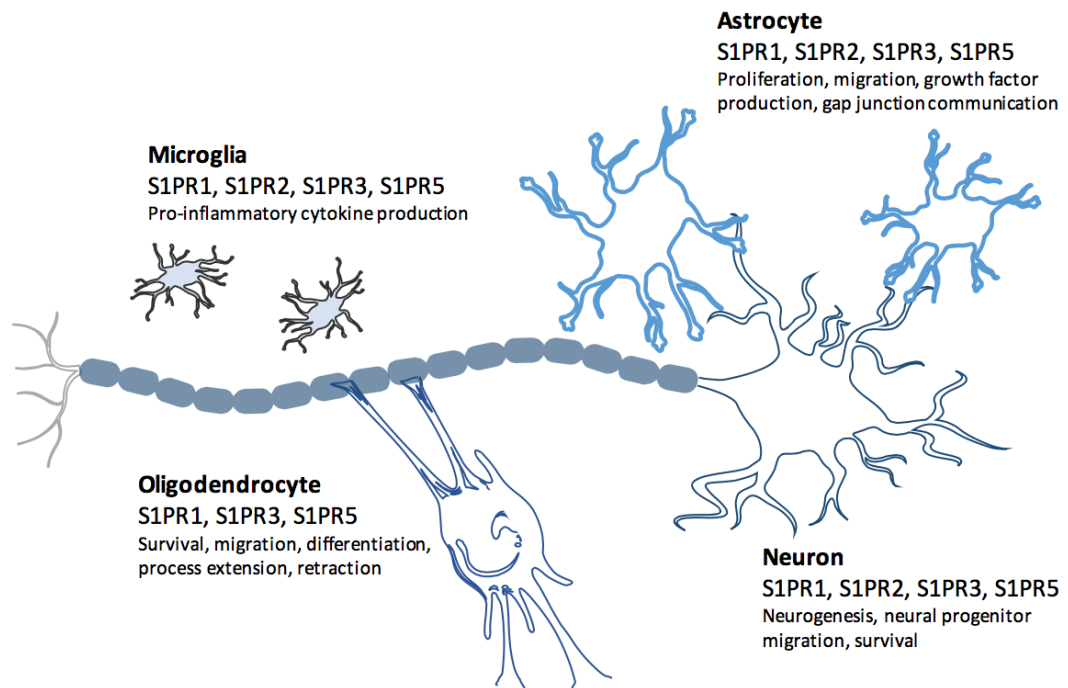


Figure 1.5 Relative expression of S1PRs in various cell types of the CNS

Astrocytes have an S1PR expression pattern of S1PR3>S1PR1>S1PR2>S1PR5. Neurons have a relative prevalence of S1PR3>S1PR1≈S1PR2≈S1PR4. Microglia's S1PR expression is mostly to S1PR1>S1PR3>S1PR2>S1PR5, and, finally oligodendrocytes mostly express S1PR5>S1PR1 (**Choi and Chun 2013**).

Ligand	Receptor	Chemical Structure	Refs
S1P	S1PR ₁₋₅ Agonist		[1]
FTY720	S1PR _{1,3-5} Agonist		[1, 2]
BAF312	S1PR _{1/5} Agonist		[3]
AUY954	S1PR ₁ Agonist		[4]
CYM5442	S1PR ₁ Agonist		[5]
SEW2871	S1PR ₁ Agonist		[6]
KRP203	S1PR ₁ Agonist		[7, 8]
CS0777	S1PR ₁ Agonist		[9]
ACT128800	S1PR ₁ Agonist		[10]
RPC1063	S1PR _{1/5} Agonist		[11]
ONO4641	S1PR _{1/5} Agonist		[12]
GSK2018682	S1PR _{1/5} Agonist		[13]
MT1303	S1PR ₁ Agonist		[14]
JTE013	S1PR ₂ Antagonist		[15]
VPC23019	S1PR _{1/3} Antagonist		[16]
VPC44116	S1PR ₁ Antagonist		[17]

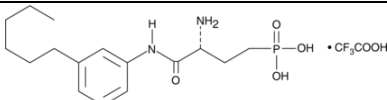
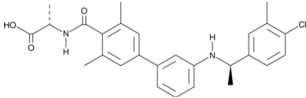
Ligand	Receptor	Chemical Structure	Refs
W146/ML056	S1PR ₁ Antagonist		[18]
NIBR-0213	S1PR ₁ Antagonist		[19]

Table 1.2 S1PR agonists and antagonists

See Appendix 1 for references.

Chapter 2 – Materials and Methods

1. Animals

All experiments were conducted in accordance with EU guidelines and protocols approved by the Trinity College Dublin ethics committee. The project was authorised by the Health Products Regulatory Authority (HPRA), under the project authorisation number AE19136/P031. A breeding colony of heterozygous mice carrying a mutation in the *galc* gene was maintained on a C57BL/6J background (Jackson Laboratories, ME, USA). Given that both fingolimod and siponimod are orally available compounds, they were administered via drinking water at a daily concentration of 1mg/kg, thus limiting exposure to unnecessary handling and harm beyond the severity of the animal model itself. Siponimod and fingolimod treatments were administered to weaned animals starting at PND21 (**Figure 2.1A**). Animals were socially housed in a temperature-controlled room under artificial illumination with a 12h light/dark cycle. All animals were given *ad libitum* access to food and water. To ease the difficulty in eating and drinking in later life, twitcher mice received diet gel or crushed food pellets on the cage floor. Twitcher mice were humanely euthanized should they display a 20% loss of total body weight, severely impaired mobility with paraplegia, or if deemed moribund with inactive behaviour accompanied by severe twitching and weight loss.

The neonatal pilot study was approved by the HPRA, under the amended project authorisation number AE19136/P031. Between PND5 and 14, pups were treated with fingolimod at a final concentration of 1mg/kg/day via suckling pipette. Animals were then decapitated, and blood was collected in EDTA coated vacuettes. Haematology analysis was carried out with a Sysmex KX-21N™. Additionally, the brain and spleen was dissected and stored at -80°C. Following promising results from the pilot study, an amended project authorisation was obtained in order to pursue the neonatal study and investigate effects of pre-weaning fingolimod administration. Fingolimod (1mg/kg/day) and vehicle (water) was administered via suckling pipette starting at PND5 (**Figure 2.1B**). This method of drug administration was maintained throughout their lifespan to ensure more accurate dosing. Housing conditions and humane endpoints remained the same as in earlier parts of the study.

2. Behavioural Analysis

2.1. Monitoring of Twitching and Mobility Scores

Twitcher mice and their healthy littermate controls were subjected to a behavioural analysis beginning at PND21 for both adult and neonatal studies. An observational scale was used to

classify the twitching severity during disease progression. Behavioural observations were taken up to three times daily and body weight was measured at each behaviour-testing time point. For twitching severity, a modified scoring system of previously published protocol was used (**Wicks et al. 2011**). Very mild and fine twitching similar to post-weaning symptoms were scored as Score 1. Mild, intermittent fine twitching was given a score 2, while constant fine twitching corresponded to a Score 3. Constant moderate twitching of body and head was given a score 4, and followed by euthanasia if scored 4 during four independent observations. Constant and severe trembling and uncontrollable twitching was the highest score, and followed immediately by humane euthanasia. See **Table 2.1** for scoring details. The locomotor deficits arising as a consequence of demyelination were quantified using a standardised scoring system that has been previously published and accepted for scoring mobility in animal models of Multiple Sclerosis (**Beeton et al. 2007**). A modified version of this scoring system was applied, with a minimum score indicating absence of disease (**Table 2.1**) (**Ripoll et al. 2011**). While this system is commonly used, it is important to note that inherent variability between researchers can occur using this approach (**Constantinescu et al. 2011**). Due to overt phenotypic emergence in twitcher mice, the genotype of animals used in this study became evident during the study, which limited somewhat genotype-based blinding. Water bottles were filled with drug by the experimenter and thus no blinding was included at the level of drug treatment. All data was randomized and analysis was performed with data blinded to both genotype and drug treatment.

2.2. Open Field Maze

Beside scoring and monitoring twitching and immobility in twitcher mice and healthy controls, the neonatal study included an Open Field Maze (OFM) test to investigate more subtle changes in behaviour at an early age and before severe immobility and twitching symptoms emerge (**Figure 2.1B**). Using the OFM added a more objective method to assess locomotion as well as anxiety-related behaviour of twitcher mice. Animals were placed in the OFM for 5 minutes at PND22 for habituation. All animals were behaviourally assessed in an OFM on PND25, PND28 and PND30 before the appearance of overtly observable immobility and twitching. The OFM apparatus consisted of a large 30cm by 40cm cage with opaque walls. Each animal was placed at the centre of the apparatus for 4 minutes. All experiments were carried out following the same protocol, at the same time of day, with same conditions of luminosity. A video camera connected to a computer was placed above the OFM. After each session, the OFM was cleaned with 70% Ethanol. Each testing session was recorded for later analysis with ANY-Maze tracking

software (Stoelting). The following parameters were analysed: distance travelled, mean speed (m/s), max speed (m/s), overall mobility, as well as time spent in the peripheral zones and in the centre of the test apparatus.

3. Genotyping

3.1. Sampling and DNA Extraction

Animals were individually identified through genotyping via polymerase chain reaction (PCR). Ear punches from all animals were obtained between PND15 and PND18 using 2mm punchers and processed immediately. Ear punches were digested in 500µl lysis buffer (1M Tris pH8, 0.5M EDTA, 5M NaCl, 20%SDS in dH₂O) containing Proteinase K (1:50 dilution) for 16-18 hours on a heat block at 55°C while rotating at 350 rpm. Following ear punch lysis, DNA was isolated through several washes in 100% and 70% ethanol. First, samples were washed in 1mL of high grade 100% ethanol and centrifuged at full speed on a bench top centrifuge for 10 minutes at room temperature. Supernatant was discarded, and samples were washed again in 500µl 70% ethanol, centrifuged for 5 minutes. Supernatant was discarded and remaining ethanol was removed with a pipette. Samples were re-suspended in 60µl of ultra-pure dH₂O and incubated for two hours at 55°C on a thermoshaker rotating at 350rpm.

3.2. Amplification of Target DNA using PCR

Amplification of DNA was evaluated by PCR directed towards amplification of the GALC gene, using the forward primer (TwIF: 5'-GCTGCTTAGAATCAATCAGACTG-3') and reverse primer (TwIR: 5'-CAAGCCATCAGTCAGAGCAAC-3'). The reaction mixture for PCR, with a total volume of 25µl, contained 2µl of purified DNA with 2mM MgCl₂, 0.4mM of each dNTP, PCR buffer, Taq DNA polymerase. Initial denaturation at 95°C for 2 minutes was followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 49°C for 120 seconds, and extension at 72°C for 40 seconds, with a final extension at 72°C for 7 minutes. Temperature cycling was run on a DNA thermal cycler (Thermocycler, Biometra).

3.3. Restriction Digest and Agarose Gel Electrophoresis

The products of amplification were subjected to restriction enzyme digestion for 2 hours at 37°C, before being separated on a 3% agarose gel (Sigma, A9539) in 1x TAE buffer containing

8µl of nucleic acid dye (PAGE GelRed, Biotium, 41008-T). Samples were run at 100V for 80-90 minutes and results were read under an UV light (Syngene GVM20 GelVue UV light box).

4. Tissue Harvesting

Mice were euthanised by placing in a CO₂ chamber. The spleen and kidneys were removed and stored in Radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (cOmplete, Roche, 11836170001). Mice were then transcardially perfused with ice-cold PBS. Once perfusion was complete, animals were decapitated and brains were removed. One hemisphere was split into cortex and cerebellum, which were separately kept in RIPA buffer and protease inhibitors at -80°C. The other half of the brain was post-fixed in 4% PFA (24 hours over night) before cryoprotection in 30% sucrose solution at 4°C (see **Figure 2.1C**). Brains were then snap-frozen in isopentane on dry-dry ice and stored at -80°C until further use. Spleen, kidney and spinal cord were dissected and stored in RIPA buffer containing protease inhibitors at -80°C until further use. Sciatic nerves were dissected and either stored in RIPA buffer with a protease inhibitor cocktail to be used for western blots, or processed for whole mount staining (see **Figure 2.1C**).

5. Histological Staining

Luxol Fast Blue/Periodic Acid-Schiff (LFB/PAS) staining was performed on 12µm cerebellar slices in order to examine and quantify PAS-positive infiltrating macrophages in demyelinated lesions. Sections were rehydrated with deionised water and incubated in LFB overnight at 37°C. Following washes with 70% ethanol and distilled water, sections were differentiated in 0.05% lithium carbonate for 10 seconds and immediately rinsed in distilled water. For the staining of globoid cells, sections were placed in Periodic Acid solution for 5 minutes at room temperature and rinsed with water. Following a 15-minute incubation in Schiff Reagent to achieve desired contrast, sections were washed in lukewarm tap water dehydrated in two changes of anhydrous alcohol. Finally, sections were cleared with Histo-Clear and mounted.

6. Immunohistochemistry

Frozen cerebellum hemispheres were placed in optimal cutting temperature compound (OCT) and cut sagittally at 12µm thickness. Sections were stored at -80°C until further use. Prior to immunofluorescent staining, cryosections were rehydrated with PBS. Slices were then

permeabilised using 0.1% Triton X-100 (Tx) in PBS and blocked with 10% bovine serum albumin (BSA) at room temperature for 3-4 hours. Primary antibody incubations were conducted overnight at 4°C in PBS containing 2% BSA and 0.5% Tx. Secondary antibody were carried out over 48 hours at 4°C in PBS supplemented with 2%BSA and 0.05% Tx. All primary and secondary antibodies used are listed in **Table 2.2**. Slices were mounted on microscope slides using antifade reagent and stored in the dark.

7. Light and Fluorescence Microscopy

Images were obtained using a Leica Sp8 confocal microscope and were randomly acquired throughout the cerebellum. Confocal images captured for quantitative measurement of immunofluorescent staining were 12 bit .lif files of 1024 x 1024 pixel resolution. With 5 cerebellar slices per slide, approximately 10-12 images were captured per condition at 10x, 20x and 40x magnification. Between 6 and 8 slides were used per treatment group making a minimum of 60 images analysed per treatment group. Image acquisition settings were kept the same across different treatments per experiment. Image acquisition setting were kept the same across different treatments. LFB/PAS staining was imaged using a BX51 microscope at 10x, 20x and 40x magnification". All images were saved as .tif files and were analysed manually.

8. Whole Mount Staining of Sciatic Nerves

Sciatic nerves were carefully removed and fixed in 4% PFA over night at 4°C. Following fixation, nerve samples were washed three times in PBS, each wash lasting 10mins, in a 24-well plate on an orbital shaker at room temperature. Nerves were then permeabilised and blocked overnight in 10%BSA and 1%Tx at 4°C. Primary antibodies were diluted in 10%BSA and 0.5%Tx in PBS at a 1/1000 dilution for MBP and 1/500 dilution for NFH and incubated with the nerve samples for 72hours at 4°C in an Eppendorf tube rotator. Following primary antibody incubation, nerves were rinsed in three 15-minute washes with PBS at room temperature. Nerve samples were then washed using a further 6-7 changes of PBS, with each wash lasting one hour, in order to ensure complete removal of unbound primary antibody. Nerve samples were then incubated for 48hrs at 4°C with fluorescently labelled secondary antibodies as well as Hoechst dye in 10% BSA and 1%Tx in PBS. Excess antibody was removed by washing nerve samples in PBS for 6 hours at room temperature, changing the buffer each hour. Following staining procedures, nerve samples were cleared in increasing glycerol concentrations at 25%, 50% and 75% (v/v) glycerol

in PBS with incubations lasting 12-24 hours each. The cleared nerve tissue was then mounted on a glass slide and imaged with an Sp8 confocal.

9. SDS-PAGE and Western Blot

Samples of cerebellum, spinal cord and sciatic nerve were homogenised in RIPA buffer, incubated on an Eppendorf ThermoMixer® C for 2 hours at 4°C and then centrifuged at 14.000rpm. Supernatant was equalised, mixed in 1:1 dilution with Laemmli sample buffer 2x (BioRad, 161-0737) and boiled at 90°C for 10 mins. For detection of MOG, MBP, Vimentin and GFAP 12µg of protein from each sample was loaded in duplicates onto a 12% polyacrylamide gel and then transferred to a Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010). Membranes were blocked with 5% Marvel in 0.05% PBS-Tween for one hour at room temperature and then incubated with primary antibodies overnight at 4°C. Membranes were washed with PBS-Tween (0.05%) and incubated in secondary antibodies at room temperature for 2 hours. See **Table 2.2** for antibodies used. All membranes were probed with anti-Actin anti-β-Tubulin overnight at 4°C for normalisation.

10. Statistical Analysis of Biochemical and Histological Changes

Experimental data was analysed and graphically represented using the GraphPad Prism 8.1 Software package (GraphPAD Software, Inc.). The normality of the data obtained from immunohistochemistry was established using Shapiro-Wilk normality test. Mean fluorescence intensity as measured with ImageJ software was used as an arbitrary unit of measure. Raw data sets were normalised and presented as percentages of the control group average. For that purpose, each arbitrary value in the experimental group was divided by the mean of the control group and multiplied by 100, in order to be expressed as a percentage value of the control average. Statistical evaluation of experimental data was performed with the two-way ANOVA followed by Bonferroni post-hoc test, with $p < 0.05$ as the minimum level of significance. Graphical data is represented as mean $\pm$ SEM.

11. Statistical Analysis of Behavioural Changes

Experimental data was analysed and graphically represented using GraphPad Prism 8.1 Software package (GraphPAD Software, Inc.). The difference in the lifespan of twitcher mice and healthy littermates was examined by Kaplan-Meier log-rank analysis. Discrepancies in weight

gain, twitching severity and mobility were analysed using a two-way ANOVA with Tukey's test for multiple comparisons. The normality of the data was determined using the Shapiro-Wilk test. Where appropriate, behavioural data was analysed using the parametric two-way ANOVA with the Tukey post-hoc test for multiple comparisons. If data was not normally distributed, differences between groups was assessed using the non-parametric Kruskal-Wallis followed by Dunn's multiple comparisons test. The difference in the lifespan between twitcher and wildtype mice was examined by Kaplan-Meier log-rank analysis.

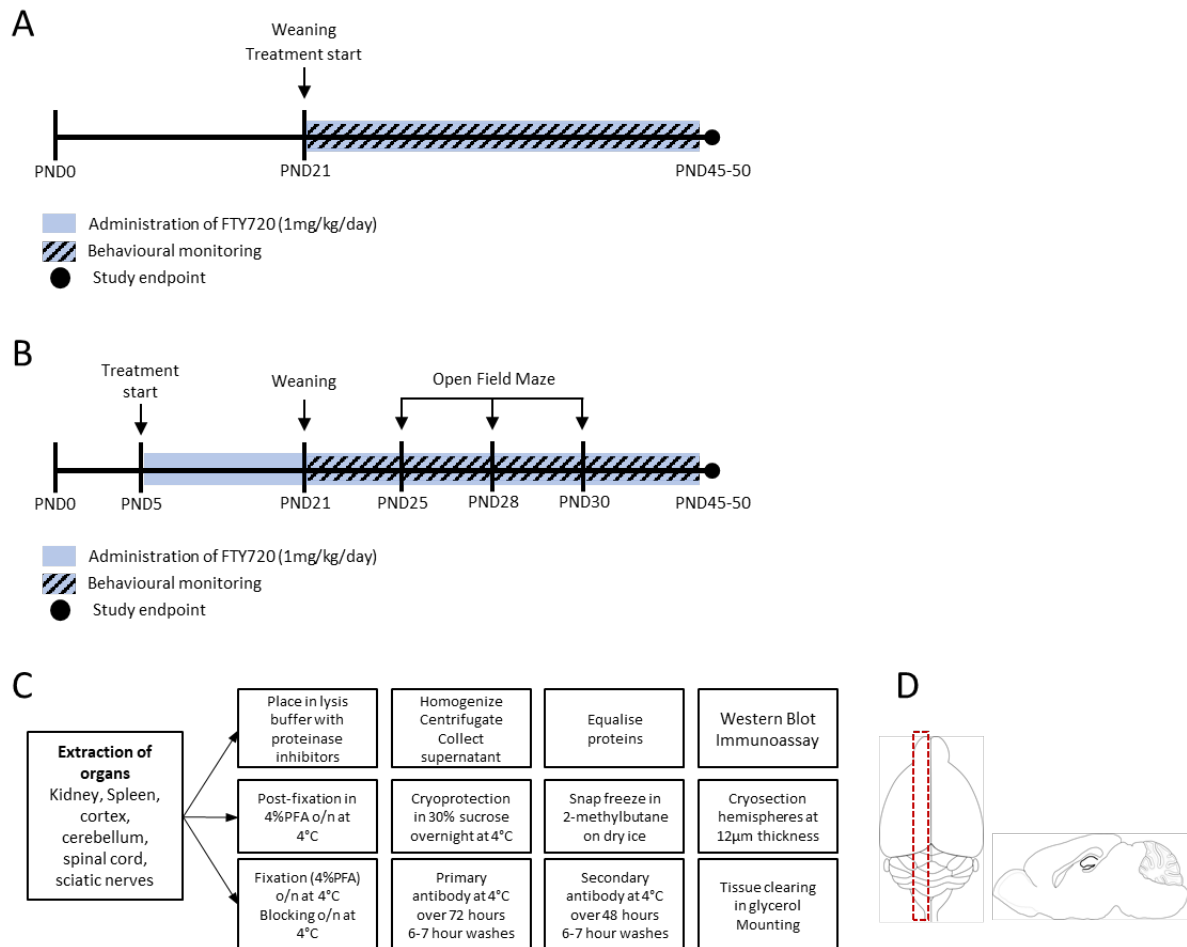


Figure 2.1 Study Design

Timelines of fingolimod treatment design for the adult (**A**) and neonatal (**B**) studies. (**A**) Animals were treated with fingolimod or siponimod (1mg/kg/day) at postnatal day 21 (PND21), via drinking water. Daily behavioural scoring of twitching, mobility and weight changes started at PND21 until animals reached humane endpoints. (**B**) Fingolimod treatment started at PND5 and was administered daily via suckling pipette until animals reached study endpoints. In addition to daily behavioural scoring, open field maze testing was conducted at PND25, PND28 and PND30. (**C**) Once animals reached behavioural study endpoints, they were humanely euthanised by CO₂ inhalation and organs were collected and processed as indicated. (**D**) Half of the hemisphere was processed for IHC, with slices taken close to the cerebellar vermis.

ANIMAL SCORE SHEET AND MANAGEMENT OF OUTCOMES

Project - Investigating new therapies for the fatal degenerative disorder Krabbe disease using the twitcher mouse model (Animal Model: Twitcher)

ANIMAL NUMBER/CODE: _____

DATE OF BIRTH: _____

EVALUATION AND RECORDING WEIGHT, TWITCHING AND MOBILITY SCORES OF ANIMAL (PLEASE USE TABLES BELOW)

With Mother / WEANING PERIOD																														MONITOR 2 x day																													
AGE (DAY)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30																													
TWITCHING SCORE*																																																											
MOBILITY SCORE*																																																											
WEIGHT OF ANIMAL																																																											
EXPECTED INCREASE IN SCORE & FATALITIES (MONITOR 3x/d)																														POSSIBLE INCREASED LIFESPAN DRUG TREATED ONLY (MONITOR 3x/d)																													
AGE (DAY)	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60																													
TWITCHING SCORE*																																																											
MOBILITY SCORE*																																																											
WEIGHT OF ANIMAL																																																											
LIFESPAN BUT POSSIBLE INCREASED LIFESPAN DRUG TREATED ONLY (MONITOR 3 x day)																														END OF STUDY																													
AGE (DAY)	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90																													
TWITCHING SCORE*																																																											
MOBILITY SCORE*																																																											
WEIGHT OF ANIMAL																																																											

BEHAVIOURAL SCORE SYSTEM AND MANAGEMENT OF ADDITIONAL OUTCOMES (SCORE 1/day & MONITOR 1-3/day)

Score	GENERAL Description	Expected Time of Score Progression; Monitoring Level	ACTION TO BE TAKEN AND HUMANE ENDPOINTS
0	None: complete absence of twitching/mobility score (pre-weaning)	Days 01-18; Monitor 1/day	n/a
x	Fatality	Days 34-46; Monitor 3/day	n/a
x	20% loss body weight over course of experiment	Days 34-46; Monitor 3/day	Euthanize immediately
x	Age 80 days (end of study)	Day 80	Euthanize immediately
Score	*TWITCHING BEHAVIOURAL Description	Expected Time of Score Progression; Monitoring Level	ACTION TO BE TAKEN AND HUMANE ENDPOINTS
1	Very mild: rare, fine twitching (post-weaning)	Days 18-22; Monitor 2/day	Allow to continue
2	Mild: intermittent fine twitching	Days 22-26; Monitor 2/day	Allow to continue
3	Constant fine twitching	Days 26-30; Monitor 2/day	Allow to continue
4	Constant moderate twitching	Days 30-42; Monitor 3/day	Euthanize upon 4th independent observation
5	Constant severe trembling and uncontrollable twitching	Days 32-46; Monitor 3/day	Euthanize immediately
Score	*MOBILITY BEHAVIOURAL Description	Expected Time of Score Progression; Monitoring Level	ACTION TO BE TAKEN AND HUMANE ENDPOINTS
1	Complete limp tail	Days 30-46; Monitor 3/day	Allow to continue
2	Mild Hindlimb paralysis	Days 34-46; Monitor 3/day	Allow to continue
3	Moderate Hindlimb paralysis	Days 36-46; Monitor 3/day	Euthanize upon 4th independent observation
4	Complete Hindlimb paralysis	Days 38-46; Monitor 3/day	Euthanize immediately
5	Hindlimb and frontlimb paralysis and incontinence	Days 40-46; Monitor 3/day	Euthanize immediately
Score	MANAGEMENT OF ADDITIONAL OUTCOMES	Monitoring Time	ACTION TO BE TAKEN AND HUMANE ENDPOINTS
x	Abnormal development (twitching/mobility) during weaning period	Days 01-20 (during weaning period)	Euthanize affected animal immediately
x	Moribund state	Days 01-80 (anytime throughout experiment)	Euthanize affected animal immediately

Table 2.1 Behavioural Scoring System

Twitcher mice and their healthy littermates were monitored 1-3 times per day, starting at PND21. Their weight was measured and twitching severity and mobility impairment were manually scored during each observation. Twitcher mice reaching stages of severe twitching and immobility were euthanised by CO₂ inhalation.

Primary Antibodies					
Antibody	Host Species	Supplier	Catalogue number	Dilution	Purpose
Anti-MBP	Rabbit	Abcam	ab40390	1/1000	IHC, WB
Anti-MOG	Mouse	Millipore	MAB5680	1/1000	IHC, WB
Anti-dMBP	Rabbit	Millipore	AB5864	1/1000	IHC
Anti-NFH	Chicken	Abcam	AB5539	1/1000	IHC
Anti-Calbindin	Rabbit	Abcam		1/1000	IHC
Anti-SMI32	Mouse	Millipore	NE1023	1/1000	IHC
Anti-GFAP	Chicken	Abcam	ab4674	1/1000 1/5000	IHC WB
Anti-Vimentin	Mouse	Santa Cruz	sc-373717	1/1000	IHC
Anti-Iba1	Rabbit	Wako	019-19741	1/1000	IHC
Anti-TMEM119	Rabbit	Abcam	ab209064	1/500	IHC
Anti-beta III Tubulin	Rabbit	ECM Biosciences	TP1691	1/1000	WB
Anti-Olig2	Rabbit	Abcam	ab109186	1/2000	WB
Anti-MPZ	Chicken	Abcam	ab134439	1/1000	IHC
Secondary Antibodies					
Antibody	Host Species	Manufacturer	Catalogue number	Dilution	Purpose
Anti-Rabbit Alexa 488	Goat	Invitrogen	A11008	1/1000	IHC
Anti-Chicken Alexa 633	Goat	Invitrogen	A11008	1/1000	IHC
Anti-Mouse Dylight 549	Goat	Jackson	610-142-121	1/1000	IHC
HRP-conjugated anti-mouse	Goat	Sigma	A-89224	1/5000	WB
HRP-conjugated anti-rabbit	Goat	VWR	IMMRIR2219	1/5000	WB
HRP-conjugated anti-chicken	Goat	Invitrogen	A16054	1/10000	WB

Table 2.2 Primary and Secondary Antibodies.

Dilutions for all antibodies were optimised as necessary and used at concentrations indicated.

Chapter 3 – Fingolimod attenuates demyelination and inflammation in Twitcher mice

Chapter Aims

- Determine whether fingolimod-mediated effects are protective against demyelination observed in the CNS and PNS of twitcher mice.
- Investigate if S1PR modulation via fingolimod may be neuroprotective and/or induce neurogenesis.
- Investigate the role of S1PR modulation in regulating astrocyte and microglial activation following fingolimod administration in twitcher mice.
- Evaluate potential changes in PAS-positive globoid cell formation following fingolimod administration in twitcher mice.
- Study cerebellar purkinje cells in twitcher mice and the potential effects of fingolimod on their density.

Abstract

Krabbe's disease is an infantile neurodegenerative disease, affected by mutations in the lysosomal enzyme galactocerebrosidase, leading to the accumulation of its metabolite psychosine. We have shown previously that the S1P receptor agonist fingolimod (FTY720) attenuates psychosine-induced glial cell death and demyelination using both in vitro and ex vivo models. This data, together with a lack of therapies for Krabbe's disease, prompted the current preclinical study examining the effects of fingolimod in twitcher mice, a murine model of Krabbe's disease. Twitcher mice, both male and female, carrying a natural mutation in the GALC gene were given fingolimod via drinking water (1mg/kg/day). The direct impact of fingolimod administration was assessed via histochemical and biochemical analysis using markers of myelin, astrocytes, microglia, neurons, globoid and immune cells. The effects of fingolimod on twitching behaviour and lifespan was also demonstrated. Our results show that treatment of twitcher mice with fingolimod significantly rescued myelin levels compared to vehicle treated animals and also regulated astrocyte and microglial reactivity. Furthermore, non-phosphorylated neurofilament levels were decreased indicating neuroprotective and neurorestorative processes. These protective effects of fingolimod on twitcher mice brain pathology, were reflected by an increased lifespan of fingolimod treated twitcher mice. These in vivo findings corroborate initial in vitro studies and highlight the potential use of S1P receptors as drug targets for Krabbe's disease.

Keywords: Globoid cell leukodystrophy; Krabbe's disease; FTY720; Fingolimod; myelination, neurodevelopmental disease

1. Introduction

Krabbe's disease (globoid cell leukodystrophy) is a devastating illness that is invariably fatal within the first two years of life (**Graziano and Cardile 2015**). This disease has orphan status affecting approximately 1:100,000 births, although the incidence varies in different populations (**Barczykowski et al. 2012**). Krabbe's disease is an inherited lipid storage disorder resulting from oligodendrocyte cell death and subsequent loss of myelin. The disease is caused by mutations in the *galc* gene encoding for galactosylceramidase (GALC) (**Suzuki and Suzuki 1970**). Mutations in GALC result in enzymatic dysfunction and a build-up of its two metabolites galactosylceramide and the toxic galactolipid galactosylsphingosine (psychosine) (**Suzuki 1998**). Aggregations of the latter is particularly apparent in the white matter of the brain and in sciatic nerves, where it has been shown to inhibit some critical cell processes resulting in oligodendrocyte and Schwann cell apoptosis (**Giri et al. 2008, Misslin et al. 2017**). Pathological features of Krabbe's disease therefore include profound demyelination and almost complete loss of oligodendrocytes in the white matter, accompanied by inflammatory mechanisms including reactive astrocytosis and infiltration of numerous multinucleated phagocytes termed 'globoid cells' (**Suzuki 2003**).

The clinical phenotype of Krabbe's disease is classified based on the age of disease onset, with the majority of cases affecting infants (**Wenger et al. 2016**). Infantile Krabbe's disease typically develops within the first six months postnatal with progressive rapid neurologic deterioration. Hallmark symptoms of the classic infantile forms include irritability, hypertonic spasticity and psychomotor stagnation, followed by rapid developmental decline, seizures and optic atrophy (**Graziano and Cardile 2015**). Clinical manifestations thus suggest involvement of both the first and second motor neurons, indicative of a systemic disorder affecting the central as well as the peripheral nervous systems. To date there is no therapeutic cure for Krabbe's disease. A number of therapeutic strategies have been described, targeting various levels of the pathomechanistic cascade in order to lower the psychosine load and reduce its neural toxicity (**Bongarzone et al. 2016**). The current standard of care for patients with Krabbe's disease is limited to hematopoietic stem cell transplantations, derived from bone marrow or umbilical cord blood (**Escobar et al. 2005**). While this treatment has been shown to slow disease progression, it fails to correct peripheral neuropathy in infants (**Escobar et al. 2005, Duffner et al. 2009**). With increasing evidence suggesting Krabbe's disease to be a multi-modal illness, which includes ongoing inflammatory and neuronal pathologies, a combination of therapies targeting these processes may prove more promising.

Previously, we have shown that psychosine causes human and mouse astrocyte toxicity in culture and demyelination in mouse organotypic slice cultures, effects which were attenuated by sphingosine 1-phosphate receptor (S1PR) agonists fingolimod and siponimod (**O'Sullivan and Dev 2015, O'Sullivan et al. 2016**). S1PRs are G-protein coupled and expressed in many cell types including immune, cardiovascular and central nervous systems (**Dev et al. 2008**). The drug fingolimod targets all five S1PR subtypes, apart from S1PR₂ and is marketed as the first oral therapy for relapsing-remitting multiple sclerosis (**Kappos et al. 2015**). Fingolimod is described to work by internalising S1PRs in T-cells, thus limiting their egress from lymph nodes and dampening inflammation in multiple sclerosis (**Dev et al. 2008**). Furthermore, it has been extensively demonstrated that S1PRs regulate neuronal and glial cell function. Briefly, in glial cells, S1PRs play a role in oligodendrocyte differentiation, survival and myelination state, astrocyte cell migration, survival and cell signalling, and microglia reactivity and pro-inflammatory cytokine release (**Miron et al. 2008, Miron et al. 2010, Sheridan and Dev 2014, O'Sullivan and Dev 2015, O'Sullivan et al. 2016**).

Given that fingolimod may have potential to alter both inflammatory and neuronal dysfunction in both the brain and periphery, and having previously shown that S1PR agonists attenuate psychosine-induced cell death of astrocytes and demyelination *in vitro*, the current study aimed to examine *in vivo* effects of fingolimod in twitcher mice, the murine model of Krabbe's disease.

2. Results

2.1. Fingolimod regulates levels of myelin in twitcher animals

We have demonstrated that fingolimod promotes remyelination and inhibits demyelination in psychosine treated cerebellar slice cultures, as well as in lysophosphatidylcholine (LPC), H₂O₂ and splenocyte treated cerebellar slice cultures (**Pritchard et al. 2014, Sheridan and Dev 2014, O'Sullivan and Dev 2015**). Here, we examined myelin status in vehicle- and fingolimod-treated twitcher mice, using myelin basic protein (MBP) and myelin oligodendrocyte protein (MOG), both of which constitute late markers of oligodendrocyte maturation and are involved in the final stages of myelin compaction. As expected, fingolimod did not alter the amount of myelin in wildtype littermates under non-pathological conditions (**Figure 3.1**), an observation that coincides with previous findings (**Miron et al. 2010**). As anticipated, MBP and MOG immunostaining in vehicle-treated twitcher mice was significantly reduced compared to their wild-type littermates (MBP: WT^{Veh} 100±11.2 vs Twi^{Veh} 47.51±3.7, **p=0.0075, n=10, df=33; MOG: WT^{Veh} 100±9.9 vs Twi^{Veh} 59.97±3.4, **p=0.0036, n=8, df=26) (**Figure 3.1**). Importantly, we found the administration of fingolimod in twitcher mice increased significantly MBP expression compared to vehicle-treated animals (Twi^{Veh} 47.51±3.7 vs Twi^{FTY} 94.66±7.8, *p=0.04, n=7-10, df=33) (**Figure 3.1**), with no significant effect on MOG expression (Twi^{Veh} 59.97±3.4 vs Twi^{FTY} 82.75±3.7, ^{n.s.}p=0.248, n=7-8, df=26) (**Figure 3.1**).

The effects of fingolimod on the total levels of MOG and MBP in wildtype and twitcher mice were also examined using homogenized cerebellum (**Figure 3.2 A,B**) and spinal cord (**Figure 3.3 A,B**) tissue by Western blotting. While, the levels of MOG and MBP were reduced in twitcher mice compared to vehicle controls, in cerebellum and/or spinal cord, fingolimod did not alter the total expression levels of these markers (**Figures 3.2, 3.3**). The expression levels of Olig2, which were significantly decreased in twitcher mice compared to wildtype littermates, were also examined (**Figures 3.2C, 3.3C**). In this case, fingolimod significantly enhanced the levels of Olig2 in twitcher mice compared to vehicle-treated in the cerebellum (Olig2: Twi^{Veh} 18.209±6.1 vs Twi^{FTY} 50.684±11.8, *p=0.0386, n=9-10, df=35) (**Figure 3.1C**).

To further investigate the effects of fingolimod, we also examined levels of myelin debris, by using an antibody that recognises degraded MBP. As expected, the data showed a significant increase in levels of myelin debris in twitcher mice compared with wildtype controls (WT^{Veh} 100±7.3 vs Twi^{Veh} 171±13, **p=0.0033, n=4, df=10) (**Figure 3.4**). We note here, that the administration of fingolimod did not alter levels of myelin debris in wildtype or twitcher mice

(171 ± 13 vs Tw^{FTY} 171.3 ± 5.3 , $p > 0.9990$, $n=4$, $\text{df}=10$) (**Figure 3.4**). Overall, the data indicates that fingolimod rescues demyelination in the brains of twitcher animals via a mechanism likely involving an increase in oligodendrocyte cells expressing MBP and Olig2, with little effect on total levels of myelin or on myelin debris.

2.2. Fingolimod restores Bergmann glial cell expression and attenuates astrocyte activation

Psychosine induces death of human and mouse astrocytes *in vitro* that is attenuated by pre-treatment with fingolimod (**O'Sullivan and Dev 2015**). Here, we examined the astrocyte distribution using the type III intermediate filament astrocyte marker vimentin, which is involved in cytoskeleton formation in astrocytes, as well as examining the glial fibrillary acidic protein (GFAP), which is enhanced following astrocyte activation (**Figure 3.5**). We differentiated between Bergmann glial cells in the molecular layer (ML) and fibrous astrocytes located in the white matter (WM), as depicted by cresyl violet (**Figure 3.5**). A significant decrease in vimentin fluorescence occurred between vehicle-treated wildtype and twitcher mice in the ML of the cerebellum (ML: WT^{Veh} 100 ± 14.9 vs Tw^{Veh} 50.79 ± 4.4 , $**p=0.0131$, $n=5$, $\text{df}=14$) (**Figure 3.5**). In agreement with our previous *in vitro* data, administration of fingolimod promoted significantly the expression of vimentin in the molecular layer of twitcher mice, as compared to their vehicle-treated littermates (ML: Tw^{Veh} 50.79 ± 4.4 vs Tw^{FTY} 90.32 ± 11.3 , $*p=0.0394$, $n=4-5$, $\text{df}=14$) (**Figure 3.5**). Conversely, in the WM of twitcher mice, there was an increased vimentin fluorescence in vehicle treated twitcher mice compared to control (WM: WT^{Veh} 100 ± 27.3 vs Tw^{Veh} 239.83 ± 27.2 , $**p=0.0067$, $n=5-6$, $\text{df}=16$), which was further increased in fingolimod-treated twitcher animals (WM: Tw^{Veh} 239.83 ± 27.2 vs Tw^{FTY} 382.3 ± 24.2 , $**p=0.0033$, $n=5-6$, $\text{df}=16$) (**Figure 3.5**). GFAP immunostaining showed no significant differences in the ML or WM layers of the cerebellum of untreated or fingolimod-treated twitcher mice compared to healthy controls (WM: WT^{Veh} 100 ± 15.9 vs Tw^{Veh} 162.6 ± 20.8 , n.s $p=0.0699$, $n=5$, $\text{df}=16$; ML: WT^{Veh} 100 ± 15.8 vs Tw^{Veh} 136.644 ± 29.4 , n.s $p > 0.9999$, $n=4-6$, $\text{df}=18$) (**Figure 3.5**). Bergmann glia are generally aligned to Purkinje cell somata in ascending processes spanning the molecular layer. We noted this organised astrocyte scaffold was disrupted in twitcher mice. Notably, however, higher-magnification images of vimentin and GFAP immunostaining did not show any overt qualitative differences in fibrous astrocyte morphologies. To examine if fingolimod altered the total levels of vimentin and GFAP, Western blotting was performed using homogenized cerebellum (**Figure 3.2 D,E**) and spinal cord (**Figure 3.3 D,E**). While the levels of vimentin and GFAP increased in

twitcher mice compared to vehicle controls, fingolimod-treatment did not alter total levels of these markers (**Figures 3.2 D,E, 3.3 D,E**). Overall, these data suggest that astrocytes have an altered function in the cerebellum of twitcher mice, and this is partially modulated by fingolimod.

2.3. Fingolimod decreases microglia levels in the cerebellar white matter of twitcher mice

The CNS of patients with KD, as well as that of animal models of KD, exhibit robust neuroinflammation with microglial activation and the presence of phagocytic multinucleated globoid cells (**Potter and Petryniak 2016**). Fingolimod possesses potent anti-inflammatory effects in various animal models of CNS injury and neurodegeneration (**Aytan et al. 2016, Martinez and Peplow 2018**). Furthermore, fingolimod shifts LPS-activated microglia towards a neuroprotective microglial phenotype (**Cipriani et al. 2015**). We therefore investigated the effect of fingolimod treatment as a neuroinflammatory modulator in twitcher mice. In our hands, and surprisingly, we have not observed psychosine to alter the expression of Iba1 in organotypic cerebellar slice cultures (**O'Sullivan and Dev 2015**), although we note Iba1 is not a specific marker of altered microglia reactivity. In contrast to this, and in line with our expectations, here we found a significant increase in Iba1 immunostaining in vehicle-treated twitcher mice, as compared to their wildtype littermates ($WT^{veh} 2.675 \pm 0.5$ vs $Tw^{veh} 120.9 \pm 16$, $p < 0.0001$, $n=5$, $df=13$) (**Figure 3.6**). More importantly, fingolimod significantly decreased Iba1 expression in twitcher mice compared to vehicle treated controls ($Tw^{veh} 120.9 \pm 16$ vs $Tw^{FTY} 32.93 \pm 3.5$, $p < 0.0001$, $n=5$, $df=13$) (**Figure 3.6**). Higher magnified images of Iba1 immunostaining showed amoeboid morphology in twitcher mice compared to wildtype littermates, which appeared less so in twitcher mice administered fingolimod, although these microglia also still appeared very much of an amoeboid structure (**Figure 3.6**).

Multinucleated phagocytes are a histopathological hallmark of KD and have been shown to accumulate large amounts filamentous inclusions that stain positively for periodic acid-Schiff. The distribution of these globoid cells in the cerebellar white matter, of treated and untreated twitcher mice, was examined also by staining with luxol fast blue and periodic acid-Schiff. Our data showed increased levels of periodic acid-Schiff staining in vehicle treated twitcher mice ($1.48 \pm 0.25 \times 100 \mu m^2$), which was reduced significantly in fingolimod-treated animals ($0.75 \pm 0.05 \times 100 \mu m^2$) (**Figure 3.6**). These observations demonstrate increased Iba1 expression and

increased globoid cell expression in twitcher mice, which is attenuated significantly by fingolimod.

2.4. Effects of Fingolimod on phosphorylation state of neurofilaments and purkinje cells

Neurofilaments are neuron-specific cytoskeletal proteins essential for the development and maintenance of neurons and their processes. They form a structurally and genetically related protein subunits, which are thought to be associated with the level of myelination and to be related to the fast conduction of axons (Yuan et al. 2012). Previous studies have shown that non-phosphorylated neurofilament heavy (NFH) epitopes are expressed in neuronal cell bodies and purkinje cells under physiological conditions (Demilly et al. 2011). Increased expression of non-phosphorylated NFH in axonal tracts, however, has been associated with impaired axonal conduction, demyelination and neuronal damage (Misslin et al. 2017). To examine the NFH phosphorylation state in twitcher mice, cerebellar slices were stained for NFH and SMI-32, a marker known to label non-phosphorylated NFH. A qualitative observation showed increased expression of SMI32 in twitcher animal, as expected, which was decreased in fingolimod-treated twitcher mice, as reflected by a change in mean quantification of SMI32 fluorescence intensity. We note, however, a level of variance in the expression of SMI32 across animals, and more so in the twitcher animals compared to wildtype controls, that resulted in non-significant statistical analysis (Figure 3.7). Cerebellar purkinje cells are pivotal for motor coordination and their dysfunction generally leads to ataxia (Hoxha et al. 2018). The ataxic tremors and hind limb paralysis observed in human and murine KD suggests the pathological involvement of purkinje cells within cerebellar degeneration. Given that little is known regarding the pathology of the purkinje cell layer in KD, we investigated the number of purkinje cells in this layer by immunostaining with Calbindin. No overall difference in the number of Calbindin-positive purkinje cells was observed (Figure 3.7) in twitcher mice compared with wildtype littermates and compared to twitcher mice treated with fingolimod (Figure 3.7). Structural changes were observed, including the presence of heterotopic layers of purkinje cells of fingolimod- and vehicle-treated twitcher mice, with their soma displaced in the molecular layer (Figure 3.7). This data suggests that neuronal damage in twitcher mice is not necessarily driven by purkinje cell loss, but rather by their abnormal architectural integrity.

2.5. Fingolimod enhances lifespan of twitcher mice

The administration of fingolimod from postnatal day 21 (PND) onwards (**Figure 3.8A**), in twitcher mice, significantly improved their mobility (**Figure 3.8B**) and twitching severity (**Figure 3.8C**), which we noted had relapsing/remitting trends. Fingolimod also positively affected body weight, with significant improvement between PND25 and PND30 (**Figure 3.8D**), although this weight gain plateaued at PND30 and finally converged towards vehicle treated animals. Importantly, Kaplan-Meier survival curves demonstrated fingolimod to modestly but significantly increase lifespan of twitcher mice versus vehicle treated littermates (**Figure 3.8E**). We noted this effect showed an approximate 10% increase in average lifespan of fingolimod (42 days) versus vehicle (37 days) treated twitcher mice (**Figure 3.8E**). While we had hoped for a more promising effect on lifespan, this result reflects the well-known multi-organ involvement of leukodystrophies, where neuroprotective effects of drugs such as fingolimod may become an important part of a combination therapy that target both central and peripheral systems in these illnesses.

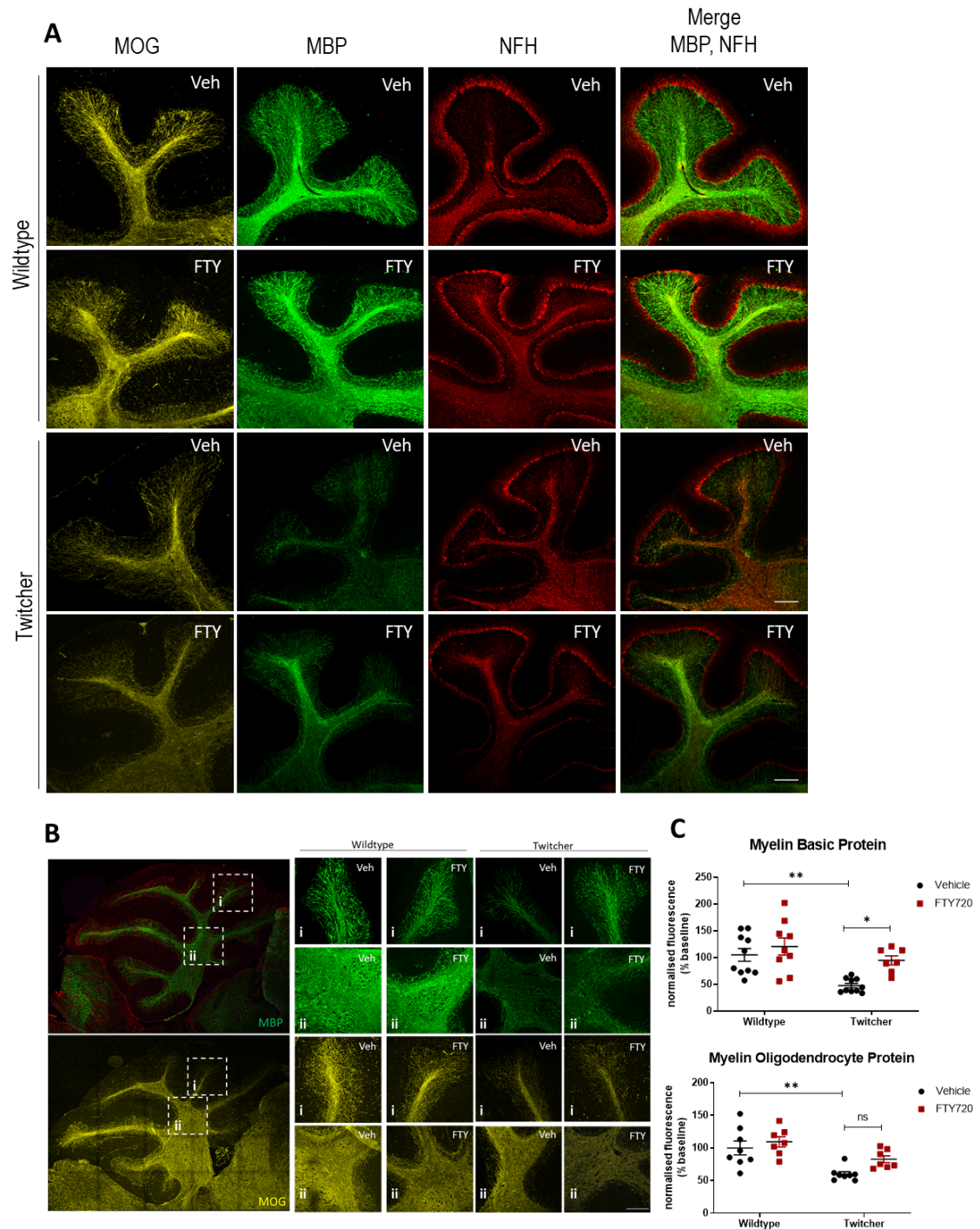


Figure 3.1 Levels of MBP in twitcher animals are rescued by fingolimod.

(A) Cerebellar slices of vehicle and fingolimod-treated twitcher and wildtype mice were processed for immunohistochemistry. Representative images display MOG (yellow), MBP (green) and NFH (red) immunostaining under treatment conditions are indicated. Confocal images were captured at 10x magnification (scale bar 200µm) with focus on cerebellar lobes. **(B)** Confocal images were captured at 20x magnification (scale bar 100µm) with focus on inner white matter and lobe VII. **(C)** Graphs illustrating changes in MOG and MBP fluorescence post treatment. Differences between treatment groups were analysed using a two-way ANOVA followed by a Bonferroni correction for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, $n = 7-10$ animals). Graphical data is represented as mean $\pm$ SEM.

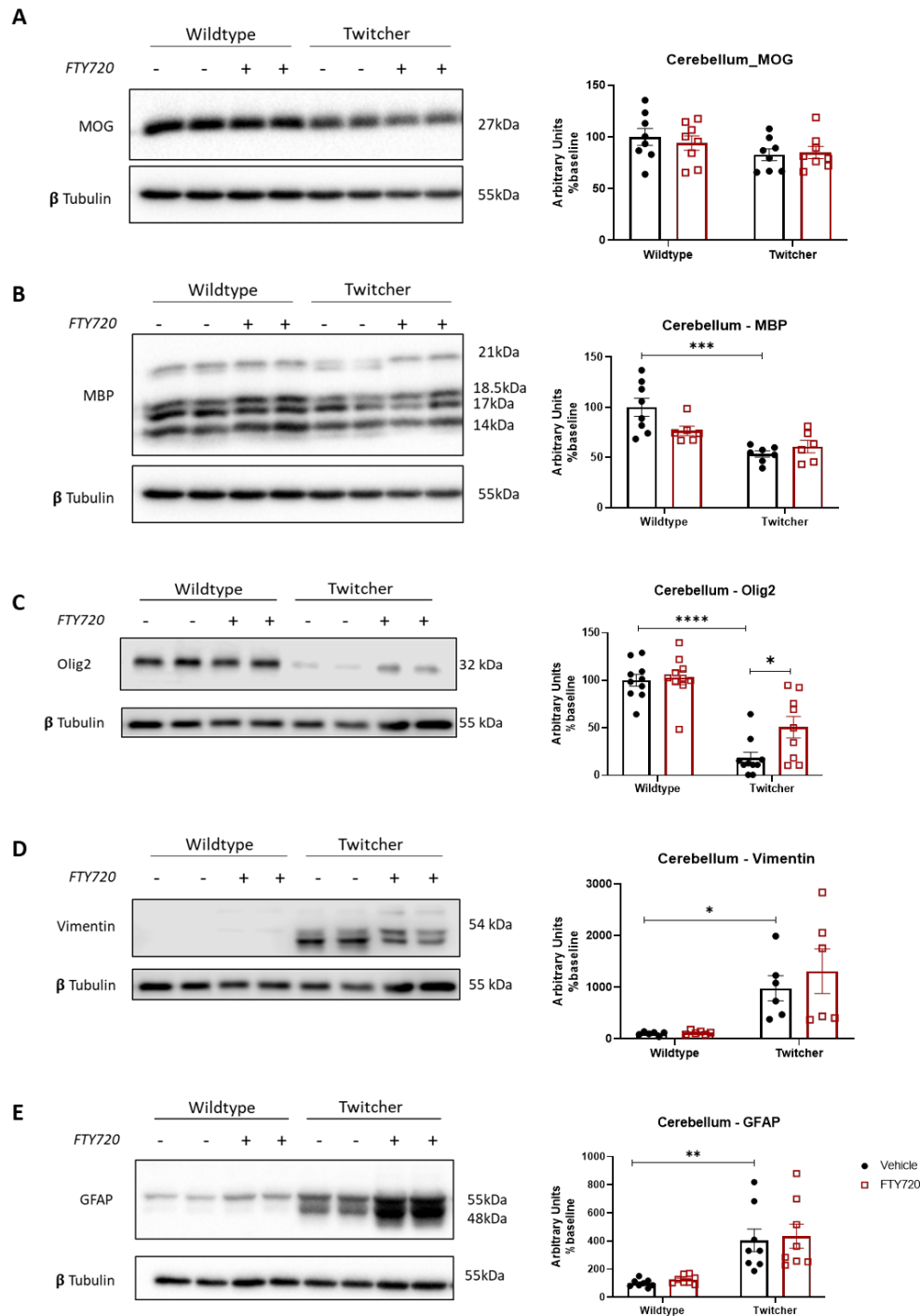


Figure 3.2 Protein changes in the cerebellum of twitcher mice.

Western blots on cerebellar tissue. **(A)** Total MOG remained at similar levels across all four treatment groups. **(B)** Data showing significant decrease in total MBP levels in twitcher mice compared to wildtype littermates, with no significant effect of fingolimod. **(C)** Olig2 was reduced significantly in untreated twitcher mice, which was increased significantly with fingolimod treatment (* $p < 0.05$, **** $p < 0.0001$, $n = 10$). Both **(D)** vimentin and **(E)** GFAP astrocyte markers were significantly increased in both treated and untreated twitcher mice. For vimentin, we noted the appearance of three independent bands, which when individually analysed followed a similar pattern as the combined total levels shown in the figure. Graphical data is represented as mean $\pm$ SEM.

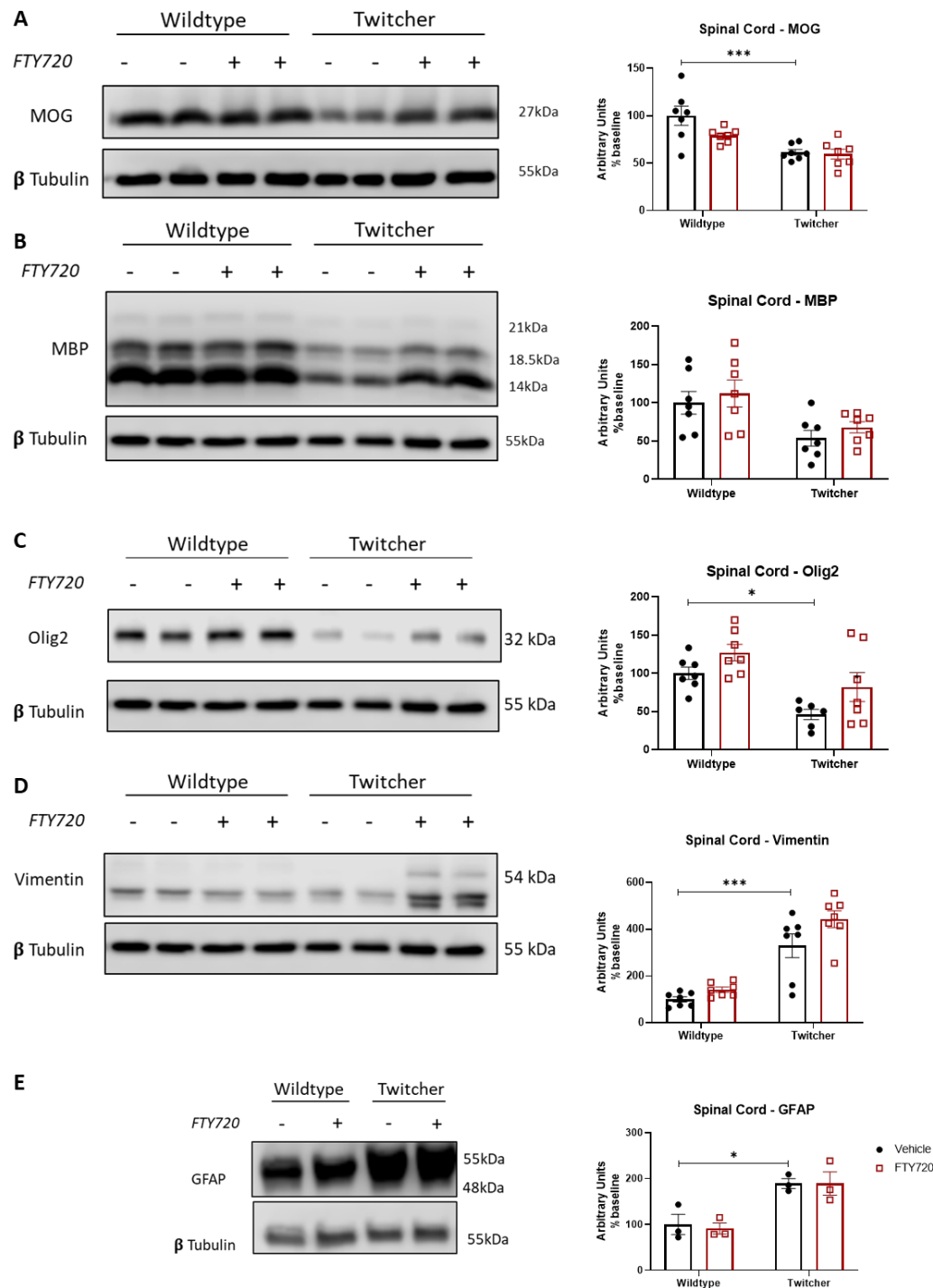


Figure 3.3 Protein changes in the spinal cord of twitcher mice.

Western blots on spinal cord tissue. Myelination as measured by **(A)** MOG and **(B)** MBP was significantly decreased in untreated twitcher mice, which was not altered with fingolimod treatment. **(C)** Olig2 was significantly reduced in untreated twitcher mice, with no significant effect in by fingolimod administration in twitcher mice (* $p < 0.05$, $n = 7$). As observed in the cerebellum, the astrocyte markers **(D)** vimentin and **(E)** GFAP were significantly increased in both treated and untreated twitcher mice. Fingolimod did not significantly change protein levels of vimentin or GFAP in treated twitcher mice. Graphical data is represented as mean $\pm$ SEM.

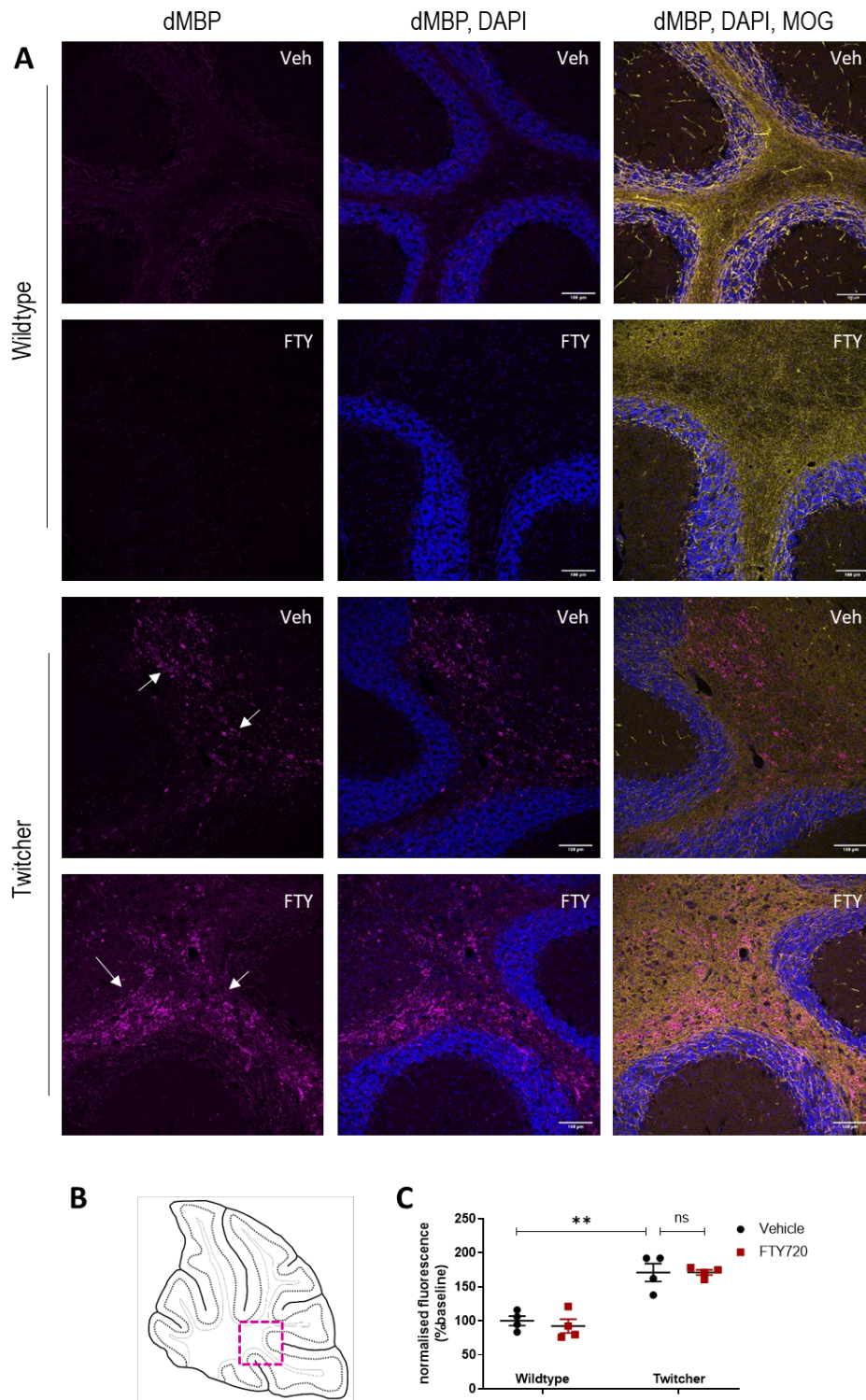


Figure 3.4 Impaired myelin debris clearance in the cerebellar white matter.

(A) Representative images showing degraded MBP (dMBP; purple), with the nuclear stain DAPI (blue) and MOG (yellow). Scale bar 100 μ m. **(B)** Images were taken at 20x magnification, in the region of the cerebellar white matter as depicted in the diagram (boxed region). **(C)** Graph illustrating changes in dMBP across treatment groups, with high levels of myelin debris in both treated and untreated Twitcher mice compared to their healthy littermates (** $p < 0.01$; two-way ANOVA with Bonferroni's *post hoc* test; $n = 4$ animals, $df = 10$).

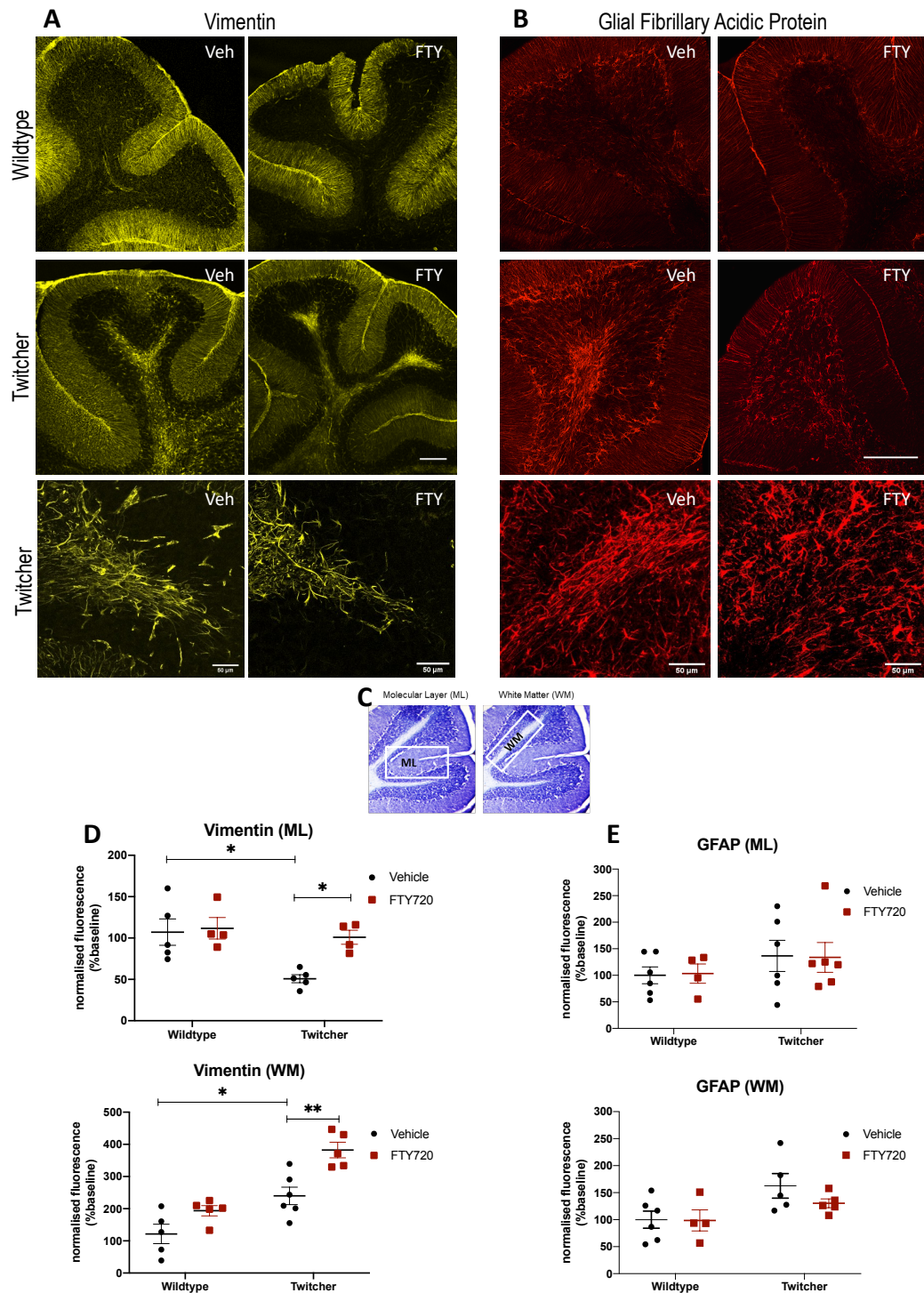


Figure 3.5 Fingolimod restores astrocyte reactivity in the cerebellum of twitcher mice.

Representative images displaying **(A)** vimentin (yellow) and **(B)** GFAP (red) in the cerebellum of wildtype and twitcher mice under treatment conditions indicated (scale bar 200µm). Magnified images of twitcher mice (lower panels) are shown (scale bar 50µm). **(C)** Cresyl stain showing regions of interest analysed, namely, molecular layer (ML) and white matter (WM). Graphs illustrating **(D)** vimentin and **(E)** GFAP expression within the ML (upper graphs) and WM (lower graphs) of the cerebellum. Data presented as mean and median $\pm$ SEM. Differences between groups were analysed using a two-way ANOVA followed by a Bonferroni correction for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, $n = 5-6$ animals).

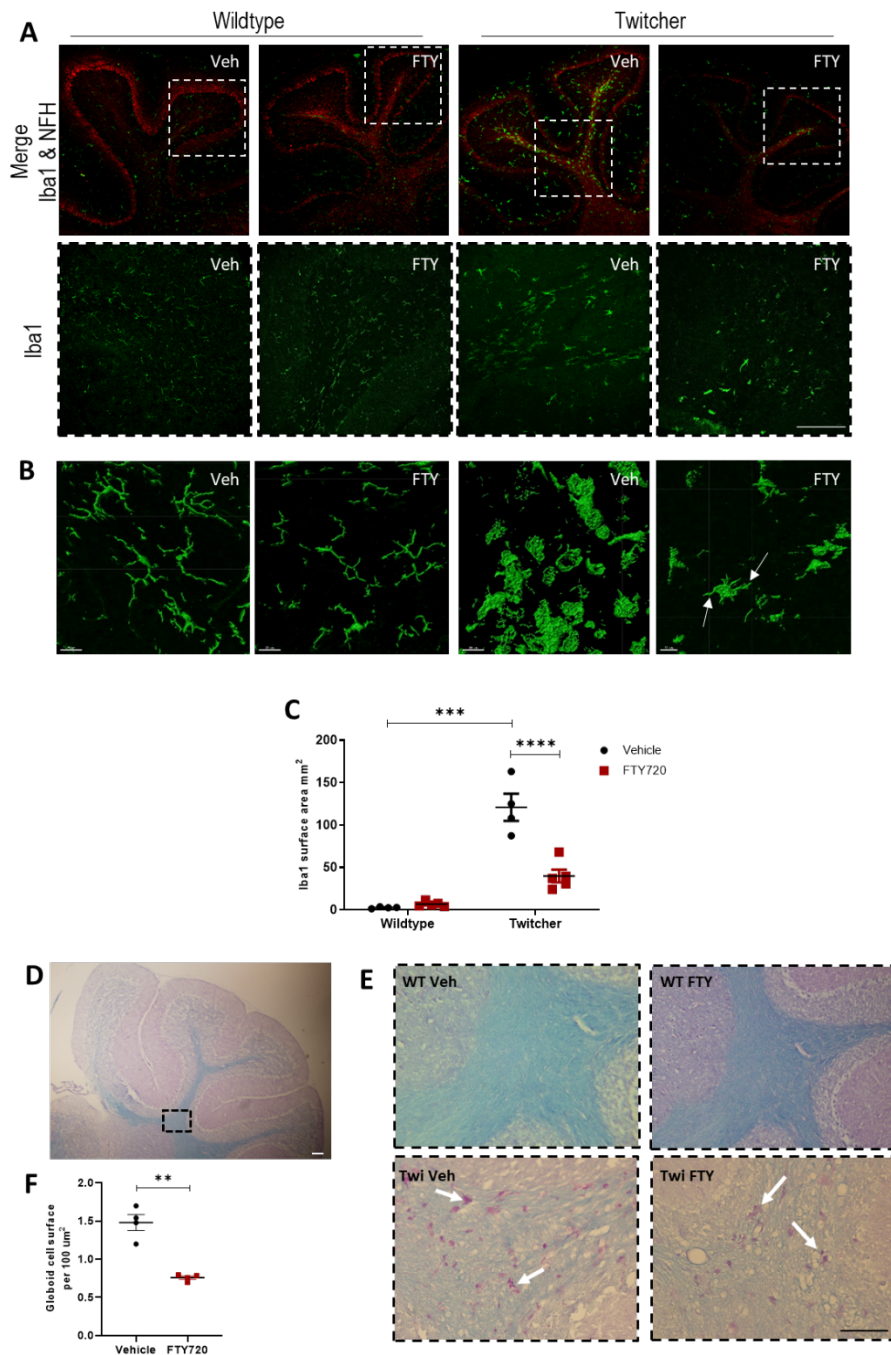


Figure 3.6 Fingolimod attenuates Iba1 expression in the cerebellum of twitcher mice.

Representative images of **(A)** Iba1 (green) and NFH (red) immunostaining. Iba1 was significantly increased in untreated twitcher mice, with accumulations centred around the white matter. **(B)** Magnified images created using IMARIS software are shown, (scale bar 20µm). **(C)** Graph illustrates Iba1-positive areas expressed as mm². Data presented as mean ± SEM. Differences between treatment groups were analysed using a two-way ANOVA followed by a Bonferroni correction for multiple comparisons (n=4-5 animals). Scale bar 200µm. **(D)** Low magnification representative image of PAS stained globoid cells (purple) accumulating along white matter tracts (blue) **(E)** High magnification representative images of PAS stain, from region indicated in dotted box in (E) (scale bar 200µm). **(F)** Graph illustrates globoid cell surface per 100µm² in twitcher mice treated with vehicle or fingolimod. Differences analysed with an unpaired t-test, p<0.01 (n=4 animals). Graphical data is represented as mean ± SEM.

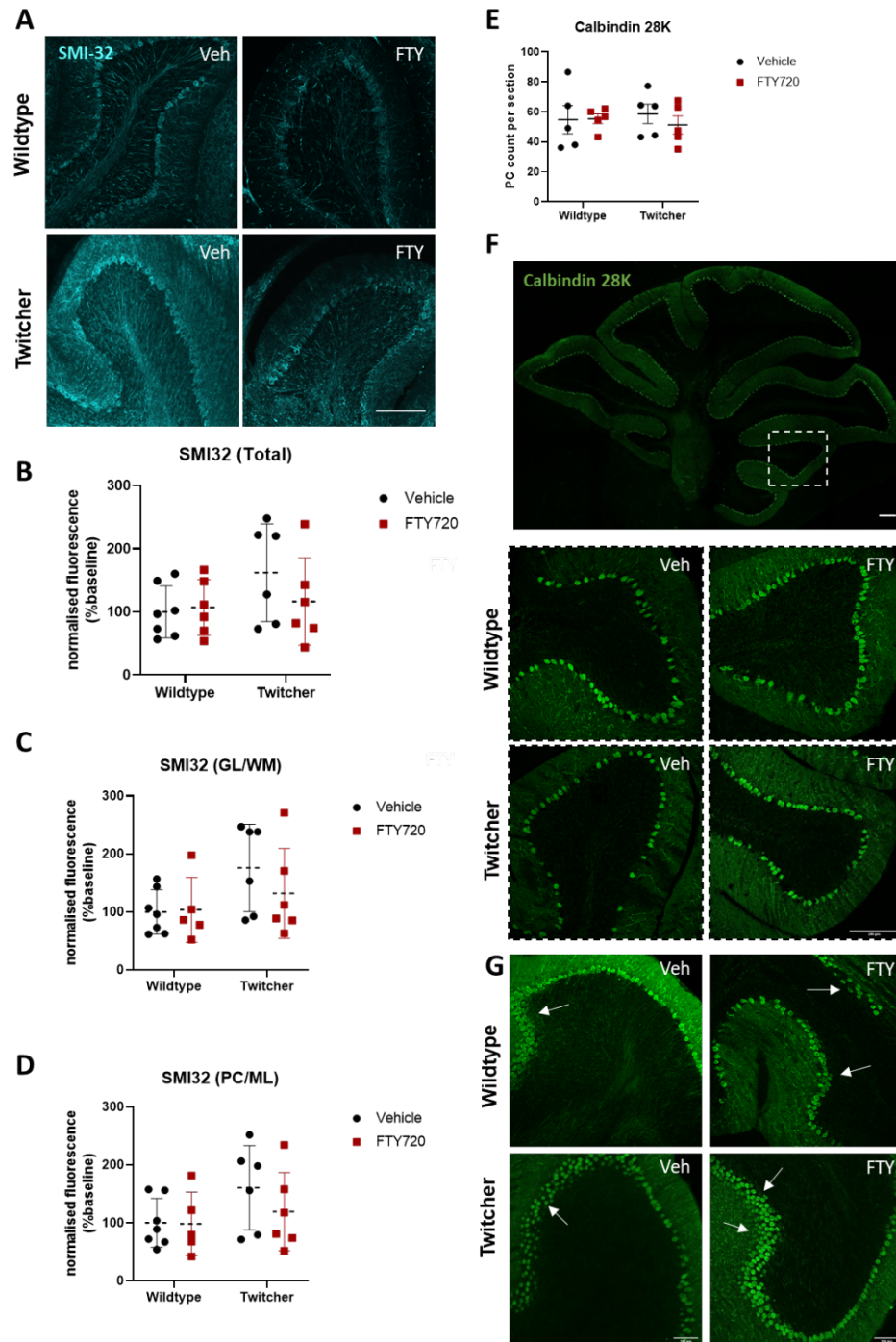


Figure 3.7 Fingolimod did not alter axonal damage or Purkinje cell layer architecture in twitcher mice.

(A) Representative images of SMI32 immunostaining. Confocal z-stacks were captured at 20x magnification (scale bars 200µm). **(B-D)** Graphical data showing SMI32 fluorescence intensity measurements, which were taken across the different layers of the cerebellar cortex (n=6-7 animals). **(E)** Graphical data showing Purkinje cell count did not change across treatment groups (n=5 animals). Data represented as mean ± SEM. **(F)** Low magnification representative image of Calbindin immunostaining of Purkinje cells. High magnification representative images of Calbindin immunostaining, from region indicated in dotted box. Confocal z-stacks were captured at 20x magnification (scale bars 200µm). **(G)** Representative images showing presence of heterotopic layers of Purkinje cells in vehicle- and fingolimod-treated twitcher mice and to a certain degree in healthy wildtype animals (scale bars 100µm).

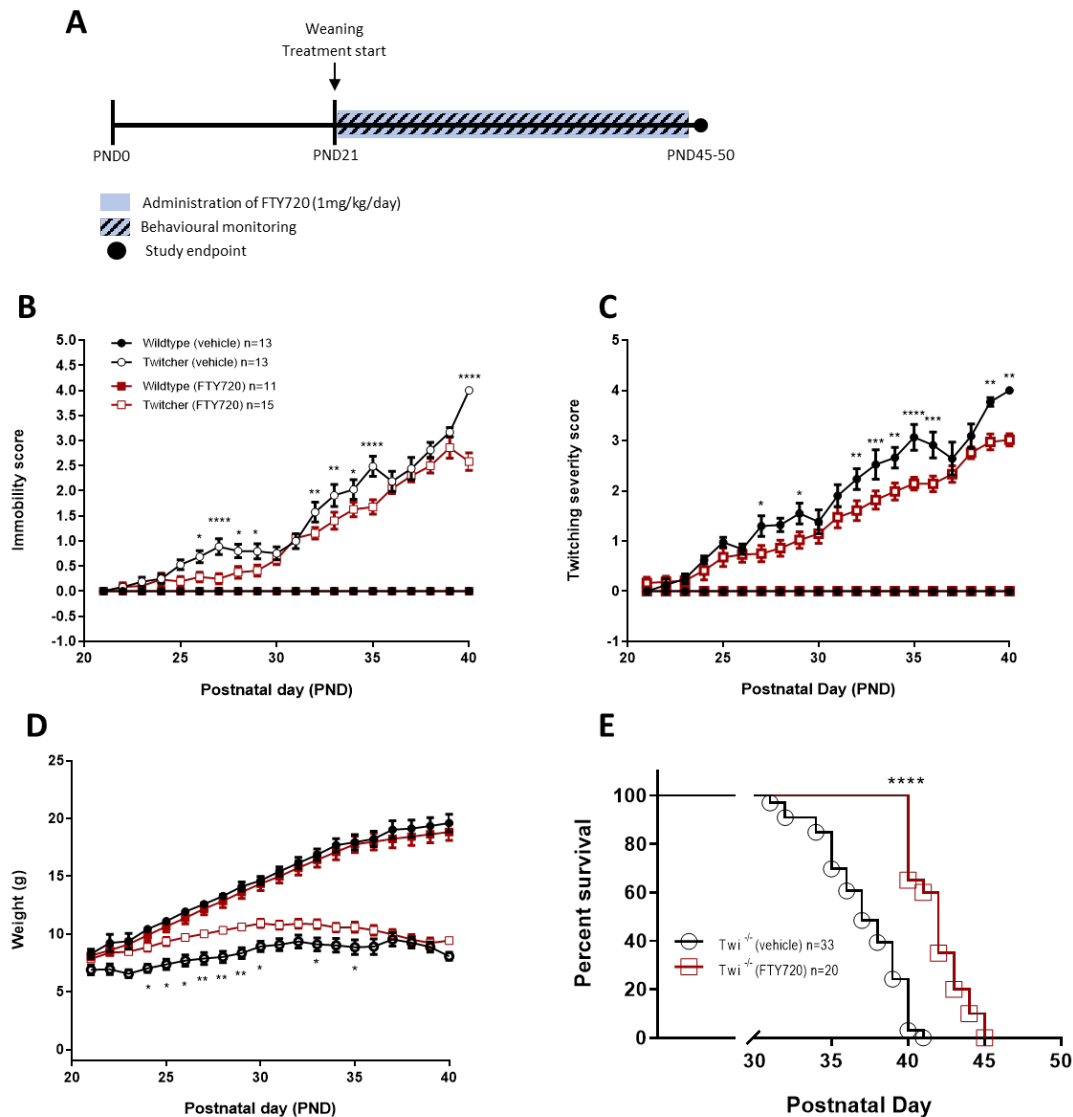


Figure 3.8 Fingolimod enhances the lifespan of twitcher mice.

(A) Schematic representation of the study design. Fingolimod-treatment commenced at PND21 at a concentration of 1mg/kg/day with daily behaviour monitoring. **(B)** Mobility, **(C)** twitching and **(D)** body weight were assessed statistically using a two-way ANOVA followed by a Bonferroni adjustment for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 11-15$ animals). **(E)** The Kaplan-Meier survival curve illustrates the lifespan of fingolimod treated versus untreated twitcher mice. A log-rank Mantel-Cox test reveals that both treatment groups were significantly different in their survival distribution (**** $p < 0.0001$, twitcher mice fingolimod treated versus untreated, $n = 20-33$ animals), with an increased average life span of fingolimod-treated twitcher mice when compared to their vehicle-treated twitcher littermates. Graphical data is represented as mean $\pm$ SEM.

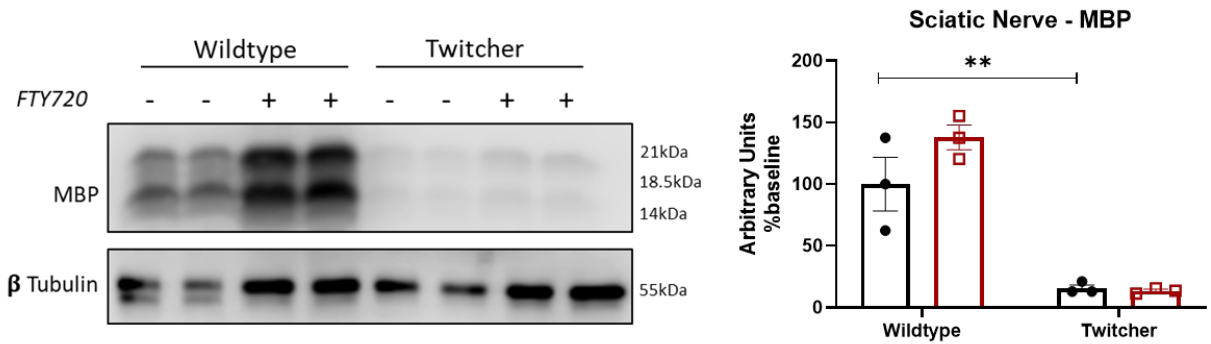


Figure 3.9. Myelin Basic Protein changes in the sciatic nerve of twitcher mice.

MBP was used as a surrogate marker for measuring myelin levels in the sciatic nerves. As expected, there was a significant decrease of MBP levels in untreated twitcher mice ($p=0.0068$, $n=3$), which was not altered by fingolimod administration ($p=0.0058$, $n=3$). These findings thus suggest that fingolimod was not able to modulate peripheral nerve demyelination. Graphical data is represented as mean $\pm$ SEM.

3. Discussion

3.1. Summary of Findings

The rapid and complete loss of myelin and myelin forming oligodendrocytes is a predominant pathological feature of Krabbe's disease (KD), and is responsible for slowed nerve conduction observed in twitcher animals, a natural occurring animal model of KD (**Davenport et al. 2011**). With studies reporting remyelinating effects of fingolimod in relapsing-remitting MS (**Münzel and Williams 2013**), and based on our previous *in vitro* work demonstrating fingolimod-mediated protective effects against astrocyte cell toxicity and demyelination, we anticipated this drug to display efficacy in other demyelinating diseases, such as KD. Here, we investigated the efficacy of fingolimod treatment on both the phenotypic and underlying and biochemical abnormalities in twitcher mice, the murine model of KD. The present results enabled a direct demonstration that fingolimod can rescue deficits seen at the pathological level, namely levels of demyelination, as well as promote lifespan and attenuate phenotypic dysfunction in these animals. Effects on CNS and PNS are summarised in **Figure 3.10**.

3.2. The regulation of oligodendrocytes by fingolimod

With fingolimod being able to cross the blood brain barrier and act on S1PRs within the CNS, this drug has been postulated to enhance and stabilise myelination by direct action on oligodendrocyte lineage cells through S1PR_{1,3,5} subtypes (**Miron et al. 2010**). Specifically, mature oligodendrocytes express high levels of S1PR₅, while oligodendrocyte precursor cells (OPCs) show preferred expression of S1PR₁ (**Choi and Chun 2013**). Moreover, remyelination requires OPCs to proliferate, migrate to sites of demyelination, and finally to differentiate into mature myelin-forming oligodendrocytes, for which S1PRs play a role (**Miron et al. 2011**). The observed enhanced Olig2 expression in cerebellar samples, in conjunction with increased expression of the myelination marker MBP-induced by fingolimod may suggest a remyelination event that involves an increase in OPC numbers, although we note the caveat that Olig2 is not expressed only in OPCs. Treatment with pFTY720 regulates also the cell survival and differentiation in oligodendrocyte lineage cells in cultured rat oligodendrocytes and OPCs (**Jaillard et al. 2005, Jung et al. 2007**). Additionally, S1PR1 activation is involved in OPC mitogenesis (**Jung et al. 2007**). In a cuprizone model of demyelination, fingolimod increases the number of OPCs, without however promoting remyelination (**Kim et al. 2011**). Importantly, pFTY720 modulates mature oligodendrocyte membrane dynamics and survival responses in human oligodendrocyte

cultures (**Miron et al. 2008**). Together these findings support the possibility of an immediate protective effect of S1PR modulation on oligodendrocytes through various signalling pathways.

3.3. Fingolimod regulates microglia in twitcher mice

The *psychosine hypothesis* was introduced in 1972 and attributes the underlying cellular and biochemical pathology of KD to supraphysiological aggregations of psychosine in the periphery and CNS (**Suzuki 1998**). Emerging evidence complements this longstanding hypothesis by ascribing early demise in KD to neuroinflammation rather than myelin loss (**Nicaise et al. 2016**). In addition to fingolimod positively regulating the levels of myelin in twitcher mice, our current study supports the involvement of immunomodulation via glial cells. Activation of astrocytes and microglia have been reported to play a compounding role in disease progression of KD (**Mohri et al. 2006, Ijichi et al. 2013**). Microglia mediate inflammation through increased levels of prostaglandin (PG) D₂, while astrocytes increase PGD₂ receptor expression (**Mohri et al. 2006**). This study reported suppressed astrogliosis and reduced demyelination when blocking PGD₂ synthase in twitcher mice (**Mohri et al. 2006**). The remyelination in fingolimod-treated twitcher animals observed in our current study coincides with the reduction we observed in the immunostaining of Iba1, albeit as surrogate marker of microglia reactivity. These findings correlate with the hypothesis that regulation of S1PRs mediate enhanced levels of myelination via a mechanism that involves immunomodulatory pathways and microglia (**Ijichi et al. 2013**).

3.4. The S1P axis regulates astrocytes and astrogliosis in twitcher mice

A growing body of evidence in KD research, paints a complex picture of combined axonal, neuronal, and myelination abnormalities in the CNS and periphery, accompanied by exacerbating astrogliosis and neuroinflammatory events (**Nicaise et al. 2016, Won et al. 2016**). Dysfunctional astrocytes may be a contributing factor to disease progression of various neurological disorders (**Molofsky et al. 2012**). Astrocytes are implicated in the pathogenesis of KD, where the astrocytic expression of matrix metalloproteinase (MMP)-3 is elevated at symptomatic onset in twitcher mice, continues to rise with disease progression and potentially induces the formation of multinucleated globoid cells and activated phagocytes (**Ijichi et al. 2013, Claycomb et al. 2014**). Such findings coincide with results from our current study, where immunofluorescence in twitcher mice shows altered expression of vimentin in areas of demyelination, accompanied by PAS-positive globoid cells. Our data showed Bergmann glial cells in the molecular layer of the cerebellum stained with vimentin was reduced in twitcher mice and this was attenuated by treatment with fingolimod. In contrast, the fibrous astrocytes

populating the white matter showed increased expression of vimentin in twitcher mice, which was further enhanced by treatment with fingolimod. Both GFAP and vimentin are highly expressed in white matter astrocytes, but poorly label gray matter astrocytes (**Molofsky et al. 2012**). The differential expression of these intermediate filaments suggests that fibrous astrocytes serve a specialised function with regards to structure among myelinated fibres (**Lundgaard et al. 2014**). We note that in our study, we did not observe statistical differences with GFAP staining between wild type and twitcher mice treated with or without fingolimod. This could be explained by the fact that GFAP is expressed late in development of fibrous astrocytes, and is therefore not an adequate marker to investigate changes during early developmental stages (**Lundgaard et al. 2014**). Supporting the role of S1PRs in astrocytes, pFTY720 attenuates psychosine-induced apoptosis in human astrocyte cultures and pro-inflammatory cytokines in murine astrocyte cultures (**O'Sullivan and Dev 2015**). The selective deletion of S1PR1 from astrocytes also reduces EAE-related phenotypic severity and histological abnormalities, including demyelination and astrogliosis (**Choi et al. 2011**). These previous studies together with our own, could be interpreted as a pFTY720-induced phenotypic change in astrocytes.

3.5.Regulation of neuronal dysfunction through S1P modulation

In addition to affecting oligodendrocytes, psychosine also impairs axonal transport (**Cantuti-Castelvetri et al. 2012**). Neurons are not known to synthesise significant quantities of psychosine, which is likely transferred from glial cells, resulting in neuronal damage and impaired axonal conduction. Psychosine accumulation increases PP1 and PP2A phosphatases, inducing neurofilament dephosphorylation and inhibiting axonal transport (**Cantuti-Castelvetri et al. 2012**). Axonopathy precedes demyelination and neuronal apoptosis in twitcher mice, with neuronal damage occurring at later stages of disease progression (**Castelvetri et al. 2011, Smith et al. 2011**). Importantly, pFTY720 has protective effects on neuronal function and axonal integrity, by reducing axonal damage following demyelination, for example, in a cuprizone model or by regulating glutamateric transmission in EAE mice (**Slowik et al. 2015**). These findings coincide with our observations using SMI32 as a marker for axonal damage, where we observed a qualitative decrease in the expression of SMI32 in twitcher animals administered with fingolimod, although we note this was not reflected by a quantitative difference. Nevertheless, the current data sits in line with previous observations that suggest S1PR modulation involves a concurrent preservation of axonal damage as well as restoration of myelin levels.

3.6.S1P modulation and its impact on the cerebellar purkinje cell layer architecture

A striking pathological hallmark of KD encompasses cerebellar ataxia, found in 57% of infantile onset cases (**Debs et al. 2013**). As disease progresses, twitcher mice display tremors, ataxia and severe hind limb weakness. Such motor deterioration suggests involvement of cerebellar degeneration in addition to pyramidal tract dysfunctions. Our results displayed structural changes in the cerebellum of twitcher mice that appear to be characterised by a pruning of dendritic arborisation and heterotopic layers of purkinje cells. These findings are in agreement with other studies showing exacerbations in neuronal vulnerability and demyelination in the internal granular, purkinje and molecular layers in cerebella of twitcher mice (**Smith et al. 2014**). Heterotopic purkinje cells are typically observed in neurodevelopmental and neurodegenerative diseases, such as spinocerebellar ataxia type 1/6 or Essential Tremor, and are defined by a mislocalised cell body in the molecular layer (**Kuo et al. 2011**). In the normal cerebellum, purkinje cells form a monolayer between molecular and granular layers, with their proper anatomical location as requirement for physiological cerebellar functioning. In a disease characterised by severe cerebellar degeneration, such as KD, it is thus possible that aberrant Purkinje cell layer architecture contributes to ataxic symptoms.

3.7.Combination approaches for treatment of KD

Our results show that fingolimod treatment increased lifespan of twitcher mice by up to 5 days and intermittently attenuated phenotypic abnormalities. While disappointing, these modest improvements may be explained by ongoing disease progression in the peripheral nervous system and other visceral organs. In supplementary studies, we observed little to no improvements in sciatic nerve demyelination in twitcher mice administered with fingolimod (**Figure 3.9**). Furthermore, we noted a consistent decrease in size of visceral organs such as spleen and kidneys of twitcher mice, compared to healthy controls (data not shown). These observations imply that fingolimod-mediated improvements in the central nervous system are not sufficient to correct for the multiple organ failure, weight loss and peripheral degeneration. Weight measurements of fingolimod treated twitcher animals ultimately converges back to that of untreated littermates, and animals eventually develop tremors, flaccid paralysis of the hindlimbs that likely results from failed correction of peripheral nerve function and demyelination. In support of this, failure to correct peripheral dysfunction in KD, is considered a challenge in infants treated with pre-symptomatic hematopoietic stem cell transplantation

(Escolar et al. 2005, Wenger et al. 2016). Thus, our observations, as well as previous studies, highlight the need to target both central and peripheral systems in order to provide a more effective therapy in KD **(Lin et al. 2005, Rafi et al. 2012)**.

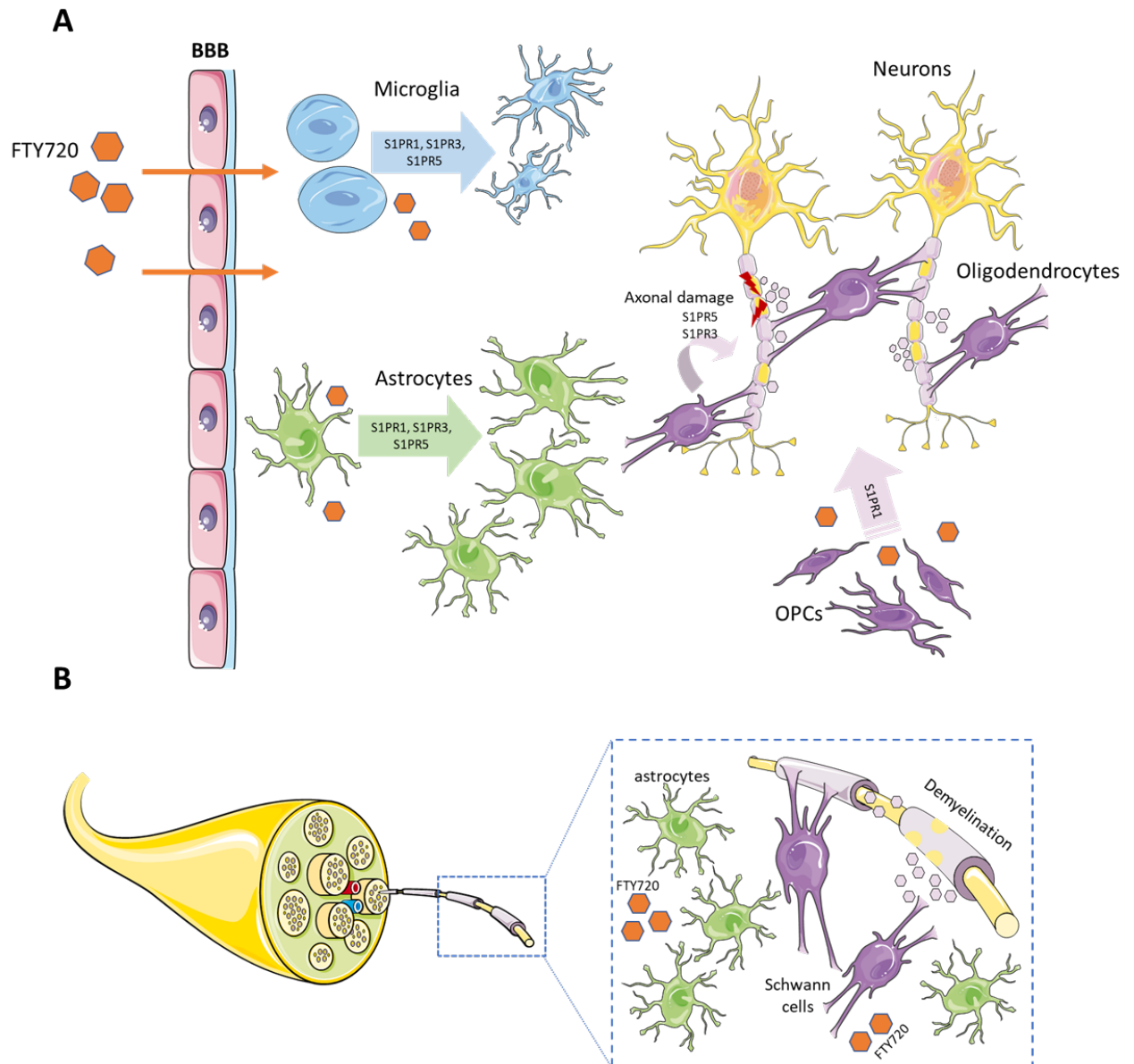


Figure 3.10 Summary figure of fingolimod-mediated effects in twitcher mice at PND21

(A) Fingolimod crosses the Blood Brain Barrier (BBB) where it acts on CNS-resident cells. We found fingolimod to significantly alter microglial morphology, from an amoeboid and reactive to a ramified phenotype. In cerebellar white matter tracts, fingolimod increased fibrous astrocytes. Importantly, here we show fingolimod was able to regulate myelination levels. Our data suggests that fingolimod effects were mediated through both immediate protective effects on mature oligodendrocytes as well as OPC recruitment, possibly via S1PR5 and S1PR1, respectively. **(B)** The severe demyelination of sciatic nerves and surrounding inflammation was not decreased by fingolimod treatment.

Chapter 4 – Neonatal administration of fingolimod does not
rescue demyelination in twitcher mice

Chapter Aims

- Examine if fingolimod can be given to pups starting at postnatal day 5 (PND5) via a pipette that triggers the suckling reflex.
- Examine whether fingolimod administered at PND5 (1mg/kg/day) reaches the peripheral blood circulation.
- Examine whether fingolimod results in adverse events when administered during weaning period and how it affects early developmental stages.
- Investigate whether beneficial fingolimod-mediated effects on behavioural abnormalities in Twitcher mice can be further enhanced when administered pre-symptomatically.
- Investigate whether fingolimod can reduce demyelination and improve remyelination in Twitcher mice.
- Investigate whether fingolimod-mediated immunomodulatory functions can regulate the inflammatory response in the brain of Twitcher mice.

Abstract

Krabbe's disease (KD) is a genetic disorder linked to mutations in the lysosomal enzyme galactocerebrosidase, which leads to the toxic accumulation of psychosine. We have shown the S1P receptor agonist fingolimod to attenuate psychosine-induced astrocyte cell death in vitro and demyelination in organotypic cerebellar slice cultures. More recently, we reported that fingolimod attenuates demyelination and glial cell reactivity in twitcher mice, a murine model of the KD. In that study, the therapeutic administration of fingolimod at postnatal day 20, modestly increased life span of twitcher mice, with neurologic symptoms such as ataxia and paralysis ultimately prevailing. Here, we hypothesized that prophylactic treatment with fingolimod, starting at postnatal day 5, may provide improved efficacy. Pathology of the cerebellum showed that early administration of fingolimod in twitcher mice reduced myelin debris, microglia reactivity and neuronal damage, while not regulating directly the levels of myelin or altering dysregulated astrocytes. In the sciatic nerve, fingolimod had little effect on myelin, Schwann cell or astrocytic cell markers. The early treatment of twitcher mice with fingolimod, while not showing efficacy on weight or locomotor assessments, was found to reduce twitching and immobility and also increase lifespan, in manner comparable to treatment at post-weaning. These findings corroborate initial studies and suggest no sizeable differences between administration of fingolimod in a prophylactic or therapeutic manner. These studies also underscore the multi-organ involvement in Krabbe's disease, and emphasise a combinatory therapeutic approach, that may include drugs such as fingolimod, to target the various mechanisms of Krabbe disease.

Keywords: Globoid cell leukodystrophy; Krabbe's disease; myelination; FTY720; fingolimod; S1P receptors

1. Introduction

The family of sphingosine 1-phosphate receptors (S1PR) are important mediators of many cellular functions, including cells in the immune and central nervous systems. S1PRs are targets of the first oral therapy of relapsing remitting multiple sclerosis, namely, fingolimod (FTY720/Gilenya®). In addition to their use in MS, S1PR drugs such as fingolimod and siponimod have been examined for efficacy in several neurodegenerative diseases, in both, pre-clinical and clinical studies (**O'Sullivan and Dev, 2017**). Given the suggested value of targeting S1PRs in a variety of neurodegenerative diseases, we examined the use of fingolimod in experimental models of Krabbe's Disease (KD). KD is a neurodegenerative globoid cell leukodystrophy that is invariably fatal within the first two years of life (**Graziano and Cardile 2015**). This genetic disease is associated with mutations in the lysosomal enzyme galactocerebrosidase, leading to the accumulation of lipid metabolites, including psychosine.

Previously, we have shown that psychosine causes human and mouse astrocyte toxicity in dissociated cell cultures and also causes demyelination in mouse organotypic slice cultures, and we have shown fingolimod attenuates these effects of psychosine (**O'Sullivan and Dev 2015, O'Sullivan et al. 2016**). Recently, we presented data that fingolimod attenuates demyelination and inflammation in twitcher mice, a murine model of the KD (**Béchet et al. 2020**). Beneficial effects of fingolimod on remyelination and inflammatory levels included a significant increase in the levels of MBP accompanied by decreases in astrogliosis and microglia reactivity in twitcher mice (**Béchet et al. 2020**). Despite promising histological findings, the therapeutic administration of fingolimod at PND21 only modestly increased life span of twitcher mice, with neurologic symptoms such as ataxia and paralysis ultimately prevailing. Given the rapid disease progression in twitcher mice, we hypothesised that early prophylactic treatment with fingolimod may yield better outcomes on lifespan. There are two key considerations regarding the use of fingolimod in very young animals that we also considered.

Firstly, the role of S1P and its receptors during early development remains to be fully elucidated. Several studies have demonstrated the importance of S1PR's in angiogenesis and vascular formation during embryogenesis (**Liu et al. 2000, Mendelson et al. 2013**). An essential role for S1P signalling in neural and vascular development has also been shown, where the disruption of the sphingosine kinase genes, *SphK1* and *SphK2*, disturbed the neural tube closure resulting in embryonic lethality (**Mizugishi et al. 2005**). Together, such findings need to be carefully reflected when targeting paediatric populations with a treatment that functionally antagonises S1P receptors.

Secondly, the pharmacodynamics and pharmacokinetic properties of fingolimod are not well studied. Clinical studies investigating pharmacodynamics show that fingolimod has a rapid onset, reducing lymphocyte counts within 4-6 hours of ingestion (**Ikeda et al. 2018**). Continued daily dosing results in the sequestration of lymphocytes in lymph nodes, with an average reduction in lymphocytes counts in the blood of 77% with a 1.25mg dose and 73% with a 0.5 mg dose. Conversely, peripheral lymphocyte increases can be observed within days of halting fingolimod intake, reaching normal counts within 1-2 months (**Kappos et al. 2006; Calabresi et al. 2014; Kappos et al. 2015; Rudnicka et al. 2015**). Fingolimod has a slow absorption period (t_{max}) of 12-16 hours, with the time of maximal concentration at 12-24 hours post dose (**Tanguay et al. 2018**). Due to a high volume of distribution, fingolimod has a half-life of approximately 9-10 days. This allows a pharmacokinetic steady state after ~6 weeks of daily fingolimod dosing, with clearance via the cytochrome P450 4F pathway (**Kovarik et al. 2004**).

Here, we present a neonatal extension study investigating the effects of administration of fingolimod at PND 5 on early developmental stages and its efficacy on lifespan in twitcher animals.

2. Results

2.1. Fingolimod reduces lymphocyte count when administered at postnatal day 5

To date, there are a limited number of *in vivo* studies that include the oral administration of fingolimod during early stages of development. A study has shown fingolimod to have neuroprotective effects in a neonatal model of cerebral white matter injury when administered via intra-peritoneal injection as a single dose (1mg/kg) at the onset of neonatal hyperoxia at postnatal day 6 (**Serdar et al. 2016**). Notably, this study reported no drug-related adverse effects (**Serdar et al. 2016**). Our present study was designed to investigate drug activity and potential side effects of fingolimod when administered orally during early development. Fingolimod was administered via suckling pipette to neonates on a daily basis between PND 5 and 14. In order to examine drug activity, and thereby confirm drug uptake, the blood from PND14 pups was processed for lymphocyte counting immediately following collection. As expected, results showed a decrease of lymphocyte numbers in fingolimod treated neonates at PND14, when compared to untreated controls (3.63 ± 0.78 n=9; 2.4 ± 0.17 n=8) (**Figure 4.1A**). In addition to evaluating lymphocyte counts, weight was measured daily between PND5 and PND14. Results show that there was a consistent weight increase over the 10-day treatment period (**Figure 4.1B**). The average weight of fingolimod-treated pups corresponded to mean weight of neonatal C57/Bl mice previously reported (**White et al. 2016**). No overt adverse effects were observed in animals receiving administration of fingolimod at PND5. Furthermore, in general, the oral administration of fingolimod, at PND5 in wildtype animals showed no changes, compared to vehicle-treatment, in the pathology or behaviour parameters discussed below.

2.2. Early administration of fingolimod regulates myelin debris, but not levels of MBP or MOG, in twitcher mice

Our previous study treating twitcher mice with fingolimod from PND21 onwards increased the expression of myelin basic protein (MBP), with no effects on myelin oligodendrocyte protein (MOG) (**Béchet et al. 2020**). Here, we evaluated if earlier treatment of fingolimod (1mg/kg/day) from PND5 onwards could enhance the myelination state in twitcher animals. Data sets were normally distributed, as shown by the Shapiro-Wilk test, allowing the use of a parametric two-way ANOVA to compare mean values between the four treatment groups. As expected, immunohistochemistry results showed that fingolimod-administration in wildtype mice did not alter the myelination state, compared to vehicle-treated wildtype mice (MOG: WT^{veh} 100 ± 4.5

vs. WT^{FTY} 99.5±4.4, n=5, ^{ns}p=0.9998) (MBP: WT^{veh} 100±13.4 vs WT^{FTY} 80.2±7.9, ^{ns}p=0.4259, n=5, df=16) (**Figure 4.2A**). Also, as anticipated, there was a decrease in MOG and MBP in vehicle treated twitcher mice compared to wildtype (MOG: WT^{veh} 100±4.5 vs Twi^{veh} 73.9±4.3, **p=0.0049, n=5, df=16) (MBP: WT^{veh} 100±13.4 vs Twi^{veh} 63.3±4.98, *p=0.0475 n=5, df=16) (**Figure 4.2A**). This decrease in MOG or MBP was not rescued by treatment with fingolimod (MOG: Twi^{veh} 73.9±4.3 vs Twi^{FTY} 80.8±5.1, ^{ns}p=0.7212, n=5, df=16) (MBP: Twi^{veh} 67.3±8.9 vs Twi^{FTY} 65.7±7.5, ^{ns}p=0.9997, p=5, df=16) (**Figure 4.2A**). Western blotting data corroborated our previous study (**Béchet et al 2020**), showing that the expression levels of MBP, but not MOG, were decreased significantly in twitcher mice, and these were not altered in twitcher mice treated with fingolimod (**Figure 4.2B**). The ability to clear myelin debris is a critical component of remyelination, a phenomenon known to be impaired in demyelinating diseases such as multiple sclerosis (**Lampron et al. 2015, Lloyd and Miron 2019**). Using an antibody against a degraded epitope of MBP (dMBP), which becomes exposed in myelin debris, we have shown previously that the administration of fingolimod, from PND21 onwards, does not alter levels of myelin debris in twitcher mice (**Béchet et al 2020**). In agreement with this previous study, the levels of dMBP were not changed between vehicle-treated and fingolimod-treated wildtype mice (WT^{veh} 100±7.2 vs WT^{FTY} 93.8±7.5, p=0.9708, n=5, df=15) (**Figure 4.2C**). Consistent with our previous study, and as expected, extensive myelin debris clusters were seen in cerebellar white matter tracts of twitcher mice compared to wildtype animals (WT^{veh} 100±7.2 vs Twi^{veh} 195.8±13.4, ****p<0.0001, n=5, df=15) (**Figure 4.2C**). Importantly, the observed myelin debris accumulation was significantly reduced by fingolimod in twitcher mice (Twi^{veh} 195.8±13.4 vs Twi^{FTY} 152.5±7.8, *p<0.0241, n=5, df=15) (**Figure 4.2C**). To summarise, we find that prophylactic administration of fingolimod from PND5 onwards reduces myelin debris with little effects on myelin, while a therapeutic administration of fingolimod from PND21 onwards increases the levels myelin with little effects on myelin debris. Taken together, this data may suggest a temporal shift from the process of myelin debris clearance to remyelination induced by fingolimod.

2.3. Early administration of fingolimod does not alter dysregulated astrocytes in twitcher mice

Psychosine-induced death of human astrocytes is attenuated by treatment with fingolimod and siponimod, *in vitro* (**O'Sullivan and Dev 2015, O'Sullivan et al. 2016**). The intermediate filamentous proteins, vimentin and GFAP, are highly expressed in astrocytes of white matter and to a lower extent in astrocytes of grey matter (**Lundgaard et al. 2014**). Using both markers,

we investigated their expression in the molecular grey matter and white matter layers, allowing analysis of Bergmann glial cells spanning the molecular layer and fibrous astrocytes lining the myelinated fibres in white matter tracts, respectively. In our previous findings, the levels of vimentin fluorescence decreased in the molecular layer (Bergmann glial cells) and increased in the white matter layer (fibrous astrocytes) of the cerebellum in twitcher mice compared to wildtype (**Béchet et al. 2020**). The administration of fingolimod at PND21 onwards attenuated the decrease in vimentin fluorescence in the molecular layer and further increased vimentin in white matter in twitcher mice (**Béchet et al. 2020**). Corroborating this initial study here, in wildtype mice, the early administration of fingolimod at PND5 onwards did not alter the expression of vimentin in either the molecular or white matter layers of the cerebellum of WT mice (ML: WT^{veh} 100±10.96 vs WT^{FTY} 105.5±12.6, p=0.9775, n=5, df=16; WM: WT^{veh} 100±18.1 vs WT^{FTY} 101.4±16.4, p>0.9999, n=5, df=16) (**Figure 3A**). Similarly, in twitcher mice, vimentin fluorescence was partially decreased in the molecular layer and increased significantly in the white matter layer of the cerebellum (ML: WT^{veh} 100±10.96 vs Twi^{veh} 80±5.7, p=0.4867, n=5, df=16; WM: WT^{veh} 100±18.1 vs Twi^{veh} 178.1±11.4, *p=0.0423, n=5, df=16) (**Figure 3A**). However, in contrast to our previous study, the early administration of fingolimod at PND5 onwards no longer altered the expression of vimentin in either the molecular or white matter layers of the cerebellum in twitcher mice (ML: Twi^{veh} 80±5.7 vs Twi^{FTY} 76.2±8.1, p=0.9916, n=5, df=16; WM: Twi^{veh} 178.1±11.4 vs Twi^{FTY} 150.9±21.9, p=0.7317, n=5, df=15) (**Figure 4.3A**). In agreement with our earlier study (**Béchet et al. 2020**), the expression of GFAP showed no significant differences in molecular or white matter layers of the cerebellum in vehicle or fingolimod-treated wildtype or twitcher mice (**Figure 4.3B**). Also in agreement with this earlier study, the total expression levels of GFAP, using homogenized cerebellum examined by Western blotting, increased in twitcher mice compared to wildtype, which was also not altered by fingolimod (**Figure 4.3C**). We again found the organised astrocyte scaffold of the alignment of Bergmann glia to Purkinje cell somata in ascending processes spanning the molecular layer was disrupted in twitcher mice. Specifically, in higher magnified images, the vimentin positive astrocytes relocated and clustered around white matter tracts, areas of demyelination and axonal damage when compared to wildtype animals. While there were no apparent differences in the morphologies of these fibrous astrocytes, fingolimod seemed to qualitatively reduce the relocated vimentin positive astrocytes (**Figure 4.3D**). Overall, these data, in best part, parallel our previous findings suggesting dysregulated astrocytes in twitcher mice, however suggest that treatment with fingolimod at an earlier time (PND5) shows limited effects compared to administration post-weaning (PND20).

2.4. Iba1 positive area in twitcher mice is diminished by early treatment with fingolimod

The sites of demyelination are usually found with associated astrogliosis, activated microglia and globoid cells. Since the development of the psychosine hypothesis, microglia have not been a primary focus in the study of KD pathology. However, sites of demyelination are usually found in conjunction with astrogliosis, activated microglia and globoid cells. To determine fingolimod mediated effects on microglia reactivity, Iba1 immunofluorescence in the cerebellum of vehicle- and fingolimod-treated twitcher and wildtype littermates was quantified. Rather than measuring fluorescence intensity, we used z-stacks to compute 3D models of Iba1-positive microglia with the software, Imaris. The surface of those 3D models was then used to identify area and volume of Iba1 positive cells, with the hypothesis that the amoeboid and reactive state of microglia in twitcher mice will be reflected in higher volume measurements. In this study, we found an expected significant increase in Iba1 volume (mm^3) and covered area (mm^2) in vehicle-treated twitcher mice (volume: WT^{veh} $48.5\text{mm}^3 \pm 6.2$ vs Tw^{veh} $77.8\text{mm}^3 \pm 7.1$, $**p=0.0061$, $n=5$, $\text{df}=16$; area: WT^{veh} $85.9\text{mm}^2 \pm 5.7$ vs Tw^{veh} $113\text{mm}^2 \pm 9.2$, $*p=0.0381$, $n=5$, $\text{df}=16$) (**Figure 4.4**). This change was reflected in the differing morphologies between the ramified resting microglia in wildtype animals compared to the amoeboid shape of the reactive microglia in twitcher mice (**Figure 4.4**). Furthermore, we noted the presence of amoeboid microglia was largely agglomerated around white matter tracts in the cerebellum, which sits in line with previous findings (**Ohno et al. 1993**) (**Figure 4.4**). While the effects of fingolimod on Iba1 volume (mm^3) did not reach significance (Tw^{veh} 77.8mm^3 vs Tw^{FTY} $56.7\text{mm}^3 \pm 7.1$, $**p=0.0538$, $n=5$, $\text{df}=16$), fingolimod significantly attenuated the Iba1-positive area (mm^2) in twitcher mice when compared to untreated twitcher mice (Tw^{veh} $113\text{mm}^2 \pm 9.2$ vs Tw^{FTY} $76.6\text{mm}^2 \pm 4.2$, $**p=0.0048$, $n=5$, $\text{df}=16$) (**Figure 4.4**). These findings strengthen our previous observations demonstrating a robust and reproducible increased Iba1 expression in twitcher mice that is attenuated significantly by fingolimod (**Béchet et al. 2020**).

2.5. Axonal damage in the cerebellum of twitcher mice is attenuated with fingolimod treatment

Neurofilaments are structural cytoskeletal proteins specific to neurons and constitute the main scaffolding for axons. They comprise four subunits namely neurofilament heavy (NFH), medium and light, as well as α -internexin (**Yuan et al. 2012**). Abnormal accumulations of neurofilaments

and aberrant phosphorylation can lead to axonal swellings, and consequentially compromise axonal transport (Louis et al. 2012). Previously, we have investigated the phosphorylation state of NFH, utilising the non-phosphorylated NFH epitope SMI-32 as a marker for neuronal damage, and have shown protective effects of fingolimod and other reagents in a variety of organotypic cerebellar slice culture toxicity models (Misslin et al. 2017, O'Sullivan et al. 2017). We have also reported a qualitative increase in expression of SMI32 in twitcher animals that is decreased by treatment with fingolimod (Béchet et al. 2020). Here, we find no significant changes in the expression levels of total NFH across the four treatment groups (Figure 4.5). In agreement with our previous study, we find SMI-32 expression in Purkinje cell and molecular dendritic layers (ML) across the four treatment groups not to change significantly, but note a higher degree of variability in the vehicle-treated twitcher mice compared to fingolimod-treated twitcher animals (Figure 4.5). Here, we observed a significant increase in SMI-32 immunoreactivity in granular layer and white matter tracts (WM) of vehicle-treated twitcher mice when compared to wildtype littermates (WT^{veh} 100 ± 15.4 vs Tw^{veh} 163.4 ± 14.4 , $**p=0.0087$, $n=5$, $df=18$) (Figure 4.5). More importantly, this increase in SMI-32 expression was significantly reduced in twitcher mice treated with fingolimod compared to vehicle-treated (Tw^{veh} 163.4 ± 14.4 vs Tw^{FTY} 115.7 ± 10.1 , $*p=0.0447$, $n=5$, $df=18$) (Figure 4.5). These findings suggest fingolimod treatment results in a certain degree of neuroprotection in twitcher animals that is perhaps more prominent when given in a prophylactic compared to a therapeutic manner.

2.6. Fingolimod administration does not alter myelin levels or astrocytic reactivity in the sciatic nerve of twitcher mice

KD is a multi-organ disease, with psychosine accumulation in multiple cell types and demyelination observed in both the central (CNS) and peripheral nervous systems (PNS). Demyelination begins during the third and fourth week in twitcher mice, and the concurrent loss of MBP in sciatic nerves has been observed at PND30 (Nagara et al. 1982, Smith et al. 2011). Given that Schwann cells express all five S1P receptor subtypes (Kohne et al. 2012), we investigated the effects of fingolimod on sciatic nerves in twitcher mice. Sciatic nerves were stained for MBP and NFH using the whole mount staining technique. In wildtype mice, high magnification images showed dense MBP fluorescence as evident by the MBP expression (green), with limited NFH staining that was presumably masked by dense myelin (Figure 4.6A). In contrast, sciatic nerves of twitcher mice displayed demyelinated axons, showing little MBP expression and primarily the remaining NFH staining (Figure 4.6A). In twitcher mice, the NFH staining was strong likely due to the nerves being severely demyelinated (Figure 4.6A). Notably,

treatment with fingolimod had no significant effects on the expression of MBP in either wildtype or twitcher mice (**Figure 4.6A**). In addition, we quantified MBP and GFAP across the four treatment groups using Western blots and also measured levels of Myelin Protein Zero (MPZ), a single membrane glycoprotein expressed by Schwann cells in the PNS. Similar to the cerebellum, data showed a significant 10-fold decrease in MBP (**Figure 4.6B**) and MPZ (**Figure 4.6C**) expression in the sciatic nerve of twitcher mice when compared to wildtype (MBP: WT^{Veh} 100 ±6.4 vs Twi^{Veh} 10.4±3.2, ****p<0.0001, n=7, df=24; MPZ: WT^{Veh} 100 ±8.98 vs Twi^{Veh} 14.1 ±2.5, ****p<0.0001, n=5, df=16), which were not changed by fingolimod-treatment in twitcher mice. Also in agreement with data found in the cerebellum, GFAP was significantly increased in the sciatic nerve of twitcher mice compared to their wildtype littermates (GFAP: WT^{Veh} 100±22.7 vs Twi^{Veh} 2204.3 ±649, **p=0.0069, n=4, df=12), which was not significantly altered with fingolimod (GFAP: Twi^{Veh} 2204.3 ±649 vs Twi^{FTY} 2088.3 ±321.9, p=0.9957, n=4, df=12) (**Figure 4.6D**). These data suggest that while fingolimod may rescue some facets of degeneration in the CNS of twitcher animals, these protective effects were not found throughout the nervous system of the twitcher mouse, which may help explain the modest efficacy of fingolimod on behaviour and lifespan, as outlined below.

2.7. Fingolimod treatment increases longevity of twitcher mice

In a previous study, we have shown that therapeutic administration of fingolimod enhances life span of twitcher mice, accompanied by improved mobility and ataxia between PND25 and PND30 (**Béchet et al. 2020**). Here, we administered fingolimod at earlier stages of development, with the hypothesis that prophylactic drug delivery could further enhance fingolimod-mediated positive effects on longevity and clinical phenotype in twitcher mice. A Kaplan-Meier survival curve showed that fingolimod significantly increased the life span of treated compared to untreated twitcher mice (**Figure 4.7A**). Additionally, fingolimod reduced twitching severity and hindlimb paralysis, with significant improvements in twitching (**Figure 4.7B**) and mobility (**Figure 4.7C**) scores starting at PND26 and lasting until animals reached humane endpoints. Despite these improvements in longevity and clinical phenotype, weight measurements of treated twitcher mice followed the same trends of vehicle treated twitcher animals (**Figure 4.7D**). We noted a decrease in size and weight of visceral organs such as spleen and kidney of twitcher mice, compared to healthy controls (Spleen: WT^{Veh} 87.2mg ±2.6 vs Twi^{Veh} 26.7mg ±3.9, ****p<0.0001, n=4, df=12) (**Figure 4.7E**) (Kidney: WT^{Veh} 193.8mg ±2.642 vs Twi^{Veh} 76.05mg ±8.461, ****p<0.0001, n=4, df=12) (**Figure 4.7F**). While there was no fingolimod-mediated effect observed in spleen weight, we report a significant increase in the weight of the right

kidney of twitcher mice (Tw^{veh} 76.05mg $\pm$ 8.461 vs Tw^{FTY} 115.825mg $\pm$ 4.3, $^{**}p=0.0028$, $n=4$, $df=12$) (**Figure 4.7F**). Overall, these observations show that the twitching, mobility, weight gain and increasing ataxia phenotypes do not differ obviously between prophylactic (PND5) and therapeutic (PND20) treatments of fingolimod. We conclude that despite fingolimod treatment resulting in some rescue of CNS and peripheral organ damage, the death of twitcher mice ensues likely due to multiple organ and systems failure.

2.8. Behavioural assessment and phenotyping of twitcher mice

In addition to behavioural assessment of twitching and immobility scoring, we applied a computer-based video-tracking system in an open field maze to detect quantitative differences in behaviour and motor activity between wildtype and twitcher mice across treatment groups. In line with our commitment to 3R animal ethics, this experiment was also conducted in the hope of identifying early signs of subtle behavioural deficits, before severity ensued. All animals were recorded at three distinct time points (postnatal days 25, 28 and 30), a time period at which the twitching symptoms and motor function of the twitcher mice remains mild to moderate. During video tracking, each animal was placed in an observational rectangular arena for video recording and manual observations for 5 minutes. Video tracking and analysis was conducted using the behavioural tracking software ANY-maze. Statistically, an ordinary two-way ANOVA revealed that the majority of open field maze parameters were linked to genotype-related differences. Multiple comparisons between the wildtype and twitcher groups were conducted using Tukey's *post-hoc* tests. If data sets were not normally distributed, the non-parametric Kruskal-Wallis test followed by Dunn's tests for multiple comparisons. The following parameters were analysed: distance travelled, mean speed (m/s), max speed (m/s), overall mobility, as well as time spent in the peripheral zones and in the centre of the test apparatus. In vehicle-treated wildtype animals, there was no change in locomotor activity parameters, except for an increased amount of time spent in the corners as the animals aged over the 6 days of testing, from PND 26 to PND28. In fingolimod-treated wildtype animals, there was a significant decrease in overall distance travelled, average speed and overall mobility over the 6 days of testing. Fingolimod treatment of wildtype animals also prevented the increase of time spent in corners as the animals aged, compared to vehicle treatment (**Figure 4.8**). These results might suggest the fingolimod reduces excitability of animals reflected by lower distance travelled, average speed, and mobility. In vehicle-treated twitcher mice, we observed a temporal decrease in locomotor parameters over the 6 days of testing (**Figure 4.8**). The fingolimod-treated twitcher mice exhibited no significant improvement any of the parameters

tested. Overall, the data points to a modest effect of fingolimod in wildtype animals, with little effect on locomotor activities in twitcher mice (**Figure 4.8**). Details about statistical tests and results for each behavioural parameter at various testing days can be found in **Appendix II**.

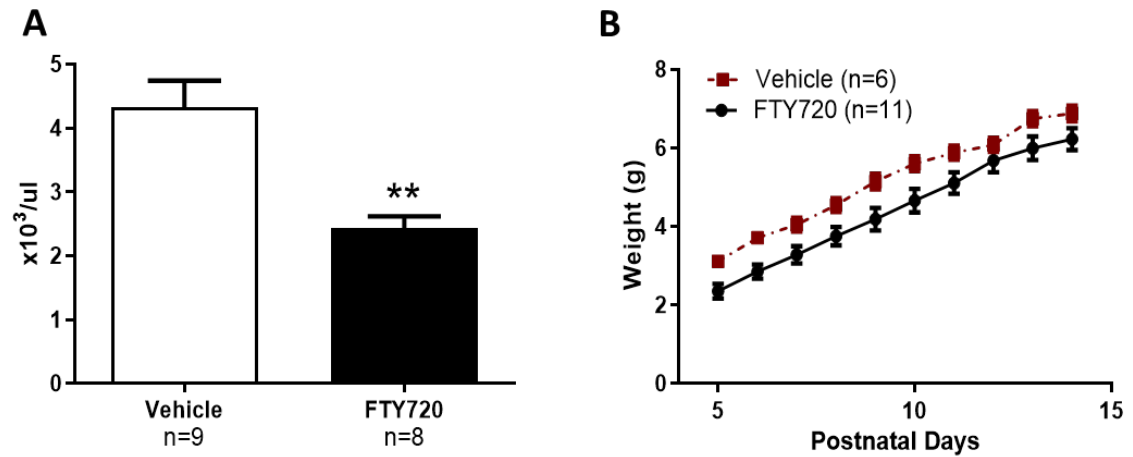


Figure 4.1 Early administration of fingolimod reduces lymphocyte counts in neonates.

(A) Lymphocyte counts were performed using the Sysmex KX-21N immediately following blood collection. Results show a decrease of lymphocyte numbers in fingolimod-treated neonates when compared to untreated control (** $p=0.0014$, two-tailed t-test). **(B)** No overt signs of fingolimod-related adverse effects were observed. Pre-weaning body weight of fingolimod-treated pups gradually increased over the 10-day treatment period, with no significant difference with vehicle treated wildtype animals.

vehicle treated twitcher mice when compared to wildtype littermates. Fingolimod did not significantly increase MBP expression as measured by densitometry. **(C)** The extent of myelin debris in the cerebellar white matter was quantified by immunohistochemistry. Cerebellar slices were immunolabelled with a marker for degraded MBP (dMBP) (purple), and fluorescence intensity was measured using ImageJ. Images were taken in the region of the cerebellar white matter as depicted in the (i) cartoon diagram (dotted box). Using an Sp8 confocal, Z-stacks were taken at 0.68 μ m intervals and flattened to give the maximum projection immunofluorescence. Graph shows a significant 2-fold increase in dMBP in twitcher mice compared to wildtype littermates, which was significantly reduced with fingolimod treatment. In all cases, confocal images taken at 20x magnification. Scale bar, 100 μ m. Data expressed as mean $\pm$ SEM, two-way ANOVA and Tukey's post-hoc test (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001). Vehicle-treated animals (black circles) and fingolimod-treated animals (red boxes).

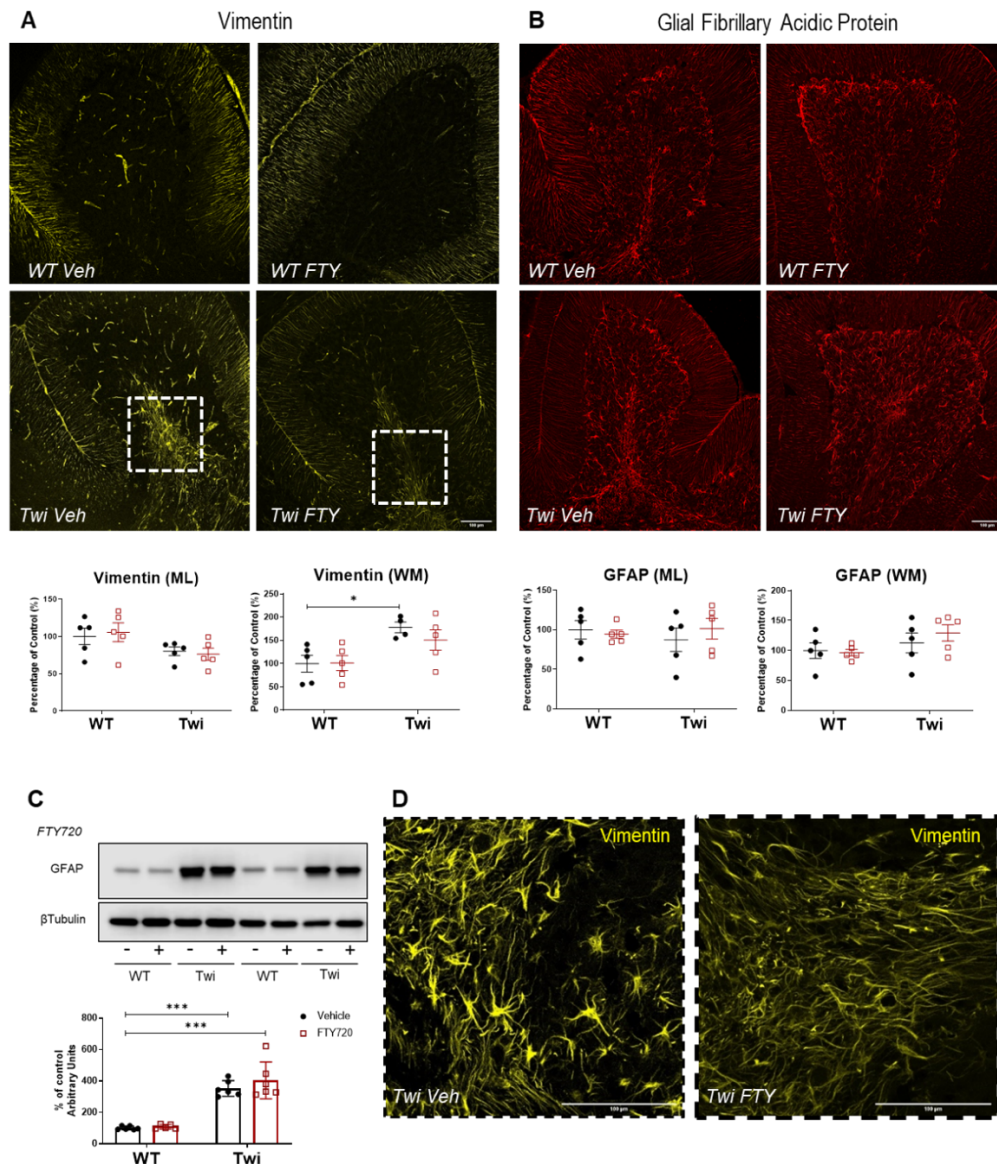


Figure 4.3 Vimentin and GFAP expression is altered in the cerebellum of twitcher mice.

(A) Representative images of fibrous astrocytes in the white matter (WM) parenchyma and Bergmann glia in the molecular layer (ML) of the cerebellum. Fluorescence intensity was subdivided into analysis of the molecular layer (ML) and the white matter (WM) tracts. Analysis of vimentin (yellow) fluorescence in the ML shows no significant differences across the four treatment groups. Within the WM tracts, vimentin labelled cells were significantly increased in vehicle-treated twitcher mice when compared to wildtype littermates. **(B)** Analysis of GFAP (red) fluorescence in the ML and WM shows no significant differences across the four treatment groups. **(C)** Western blots and densitometric analyses of GFAP immunoreactivity in cerebellar samples of vehicle- and fingolimod-treated wildtype and twitcher mice. GFAP immunoreactivity was significantly increased in both treated and untreated twitcher mice compared to wildtype, with no significant effects of fingolimod. **(D)** Higher magnified images of vimentin fluorescence taken from region of interest (dotted box) in low magnification image of vimentin fluorescence in panel (A) shows relocated vimentin positive astrocytes clustered, that were reduced by fingolimod. Confocal images taken at 20x and 40x magnification. Scale bar, 100 μ m. Data expressed as mean $\pm$ SEM, two-way ANOVA and Tukey's post-hoc test (* p <0.05). Vehicle-treated animals (black circles) and fingolimod-treated animals (red boxes).

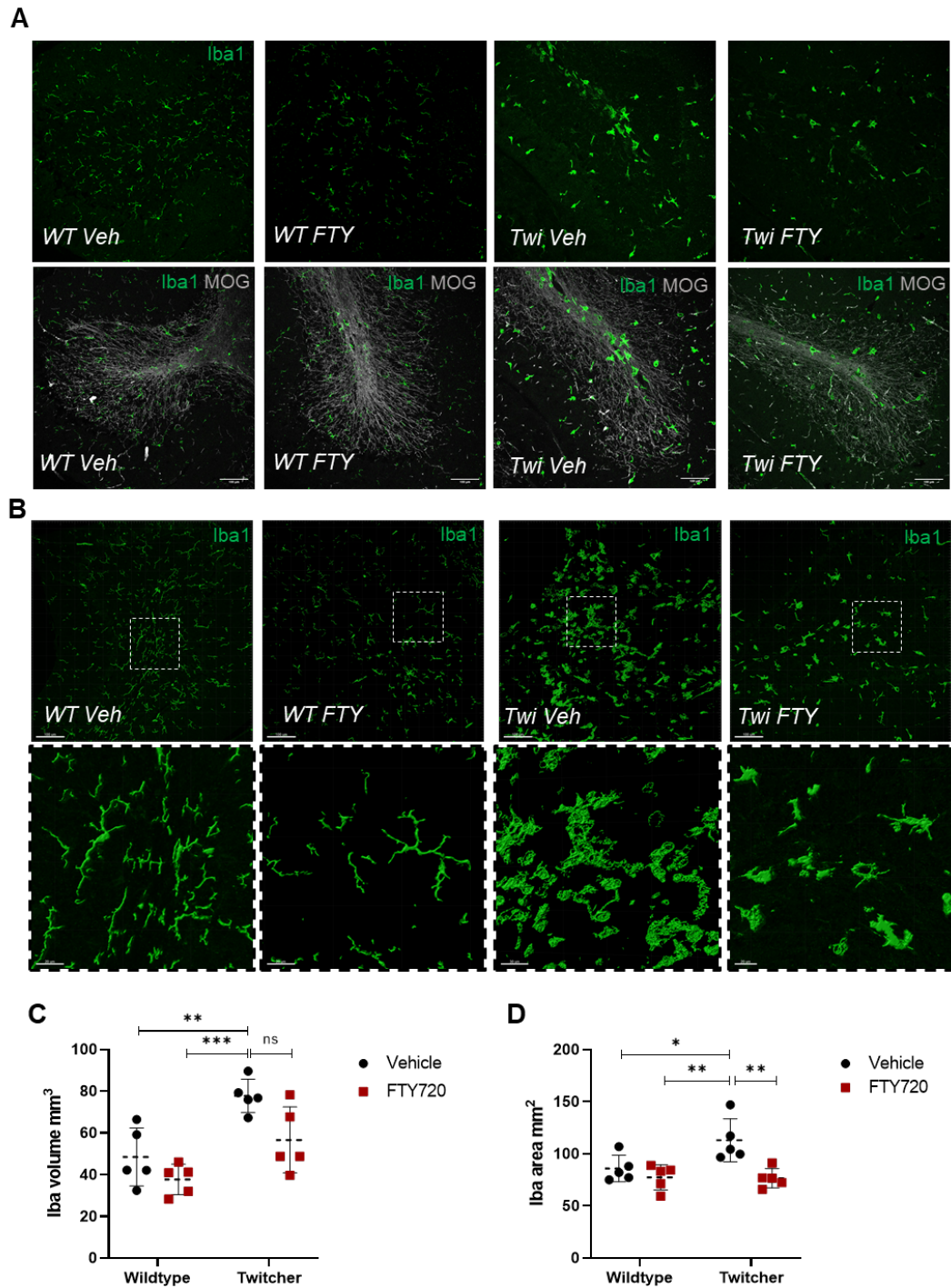


Figure 4.4 Fingolimod significantly reduces Iba1 fluorescence

(A) Representative images of Iba1-positive microglia/macrophages (green) and MOG (grey) in the cerebellum of fingolimod- and vehicle-treated wildtype and twitcher mice. In twitcher mice, Iba1-positive cells cluster around demyelinating tracts labelled with MOG (grey). (B) 3D reconstruction of Iba1 immunofluorescent microglia using Imaris bitplane. Representative images reflect the morphological changes of Iba1-positive cells in the white matter tracts of twitcher mice. Graphs showing (C) Iba1-positive volume (μm^3) and (D) Iba1-positive area (mm^2) across treatment groups. Confocal images taken at 20x magnification or 40x magnification. Scale bar, 100 μm . Scale bar inserts, 20 μm . Data expressed as mean $\pm$ SEM, two-way ANOVA and Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

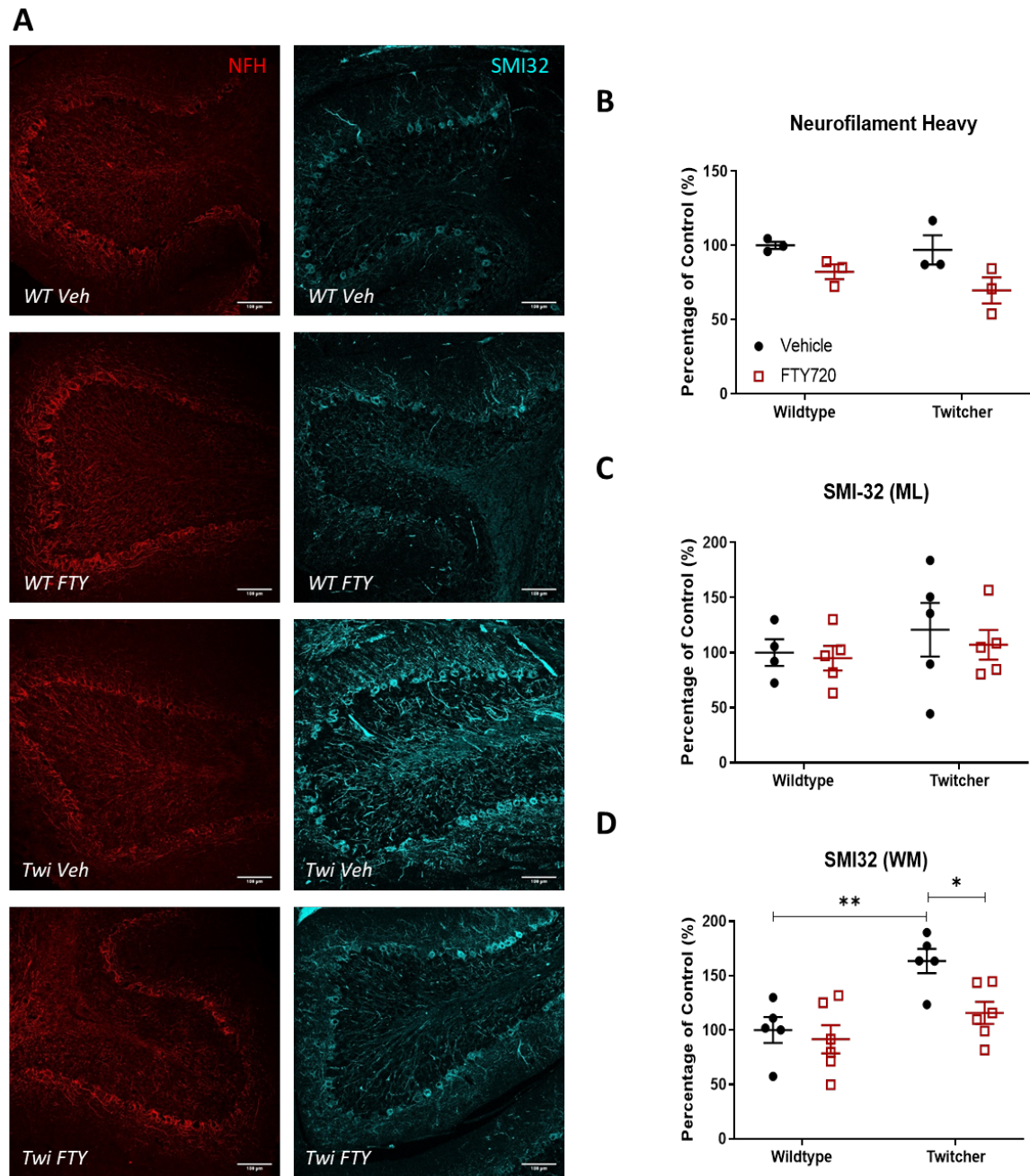


Figure 4.5 Fingolimod significantly reduces SMI-32 expression in white matter tracts of twitcher mice

(A) Representative images of NFH (red) and SMI-32 (cyan) showing changes in phosphorylated and non-phosphorylated neurofilaments in the cerebellum slices across the four treatment groups. **(B)** No changes were observed in NFH. **(C)** SMI-32 fluorescence remained similar in Purkinje cell and molecular dendritic layers (PC/ML) across treatment groups. **(D)** SMI-32 expression was significantly increased in the cerebellar granular layer and white matter tracts (GL/WM) of twitcher mice, which was reversed with fingolimod. Confocal images taken at 20x magnification. Scale bar, 100µm. Data expressed as mean ± SEM, two-way ANOVA and Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

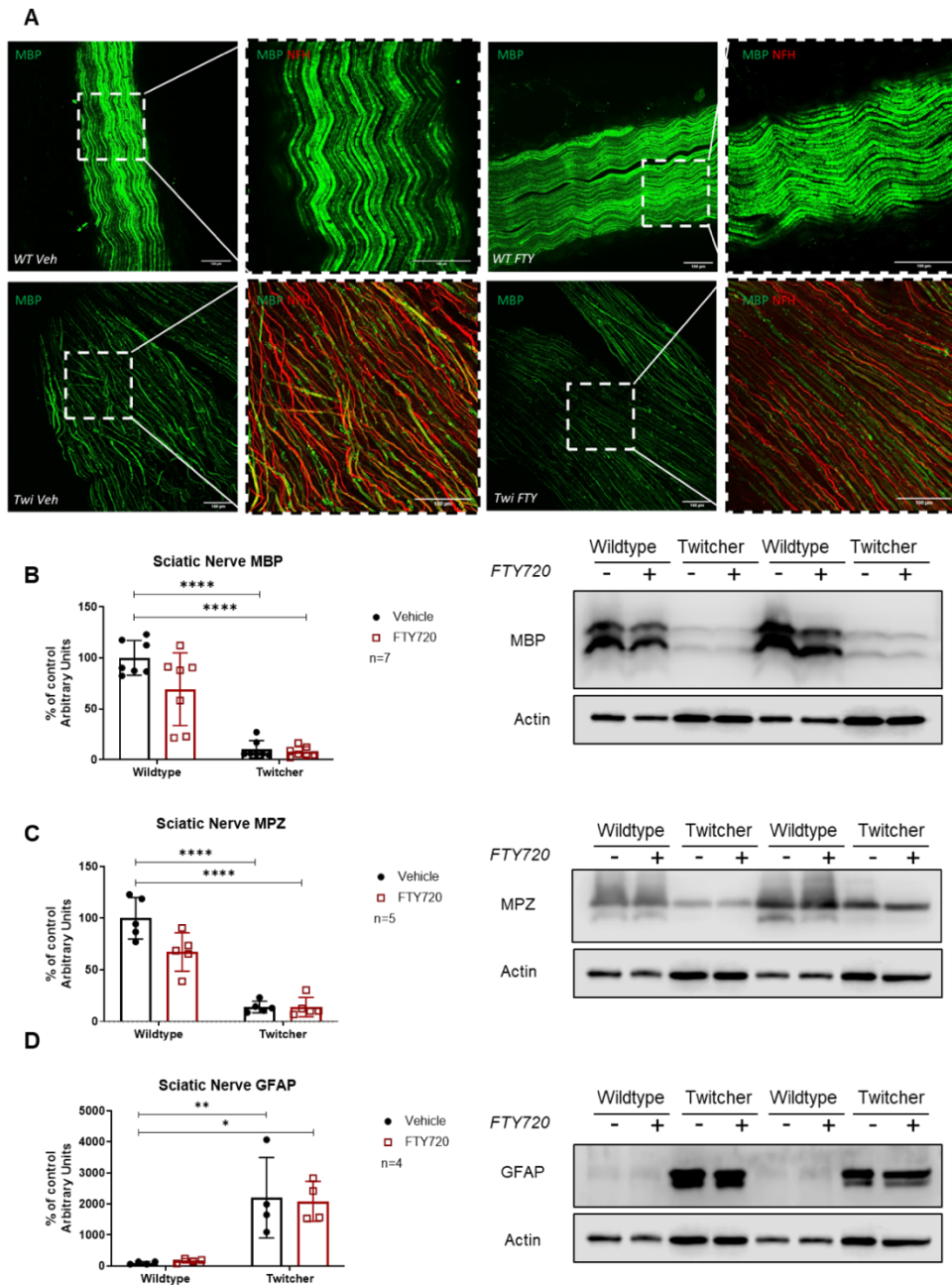


Figure 4.6. Fingolimod does not rescue demyelination or astrocyte reactivity in sciatic nerves

(A) Representative images of sciatic nerves with MBP (green) and NFH (red) immunofluorescence. Sciatic nerves were stained using a whole mount protocol, and z-stacks were captured using an Sp8 confocal microscope. The z-stacks were then flattened into a single image using the software Fiji to give a structural overview of the area of interest. Representative images show myelin fibres in wildtype animals, while sciatic nerves of twitcher mice are characterised by demyelinated axons and myelin debris. Treatment with fingolimod showed no significant changes in expression of MBP in wildtype and twitcher animals, compared to vehicle treatment. Graphs illustrating quantitative results of western blots, with markers for **(B)** MBP, **(C)** MPZ (Myelin Protein Zero, a Schwann cell marker), and **(D)** GFAP. Results show a significant decrease in MBP and MPZ in twitcher mice, that is not rescued by fingolimod. Conversely, there is a significant increase in GFAP in twitcher mice, which is not attenuated by fingolimod. Scale bar, 100µm. Data expressed as mean $\pm$ SEM, two-way ANOVA and Tukey's post-hoc test (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

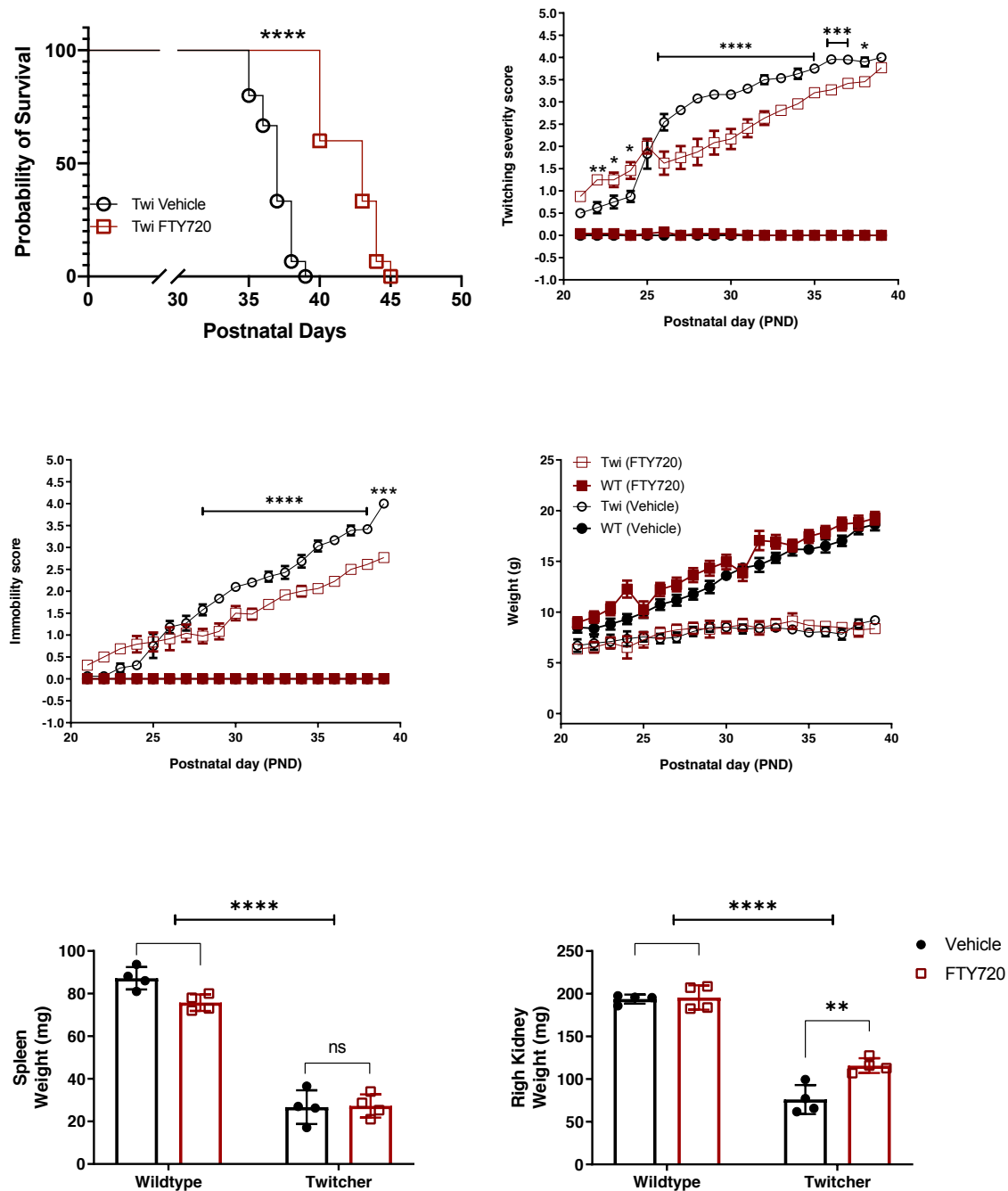


Figure 4.7 Fingolimod increases lifespan and improves clinical phenotype in Twitcher mice

(A) The Kaplan-Meier survival curve demonstrates fingolimod increases lifespan of twitcher mice compared to vehicle treated. A log-rank test reveals a significant difference in survival distribution. The (B) twitching, (C) mobility and (D) weight were statistically assessed using a two-way ANOVA followed by a Bonferroni adjustment for multiple comparisons. The number of animals per group were: vehicle (n=15) and fingolimod (n=15) treated wildtype mice and vehicle (n=13) and fingolimod (n=12) treated twitcher mice. The weight measurements of peripheral organs (E) spleen and (F) kidney show a significant decrease in size and weight in twitcher mice, as calculated by two-way ANOVA followed by a Tukey's adjustment for multiple comparisons. Notably, fingolimod treatment increased the kidney weight of twitcher animals compared to vehicle treatment. The statistical values are as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4.8 Behavioural differences within treatment groups across postnatal days 25, 28 and 30.

Following a habituation session at PND21/22, open field maze recordings were taken at PND25, 28 and 30. **(A)** Representative images of open field maze tracking plots analysed at PND25, PND28 and PND30. **(B)** Bar graphs illustrating changes between groups for each locomotor parameter studied. Vehicle treated wildtype mice did not significantly change regarding locomotor parameters tested at the three time points. Vehicle treated twitcher mice, showed a significant reduction in the overall distance travelled, the average and maximum speed which was also reflected in a reduced mobility throughout the three testing sessions. Locomotor parameters of fingolimod-treated twitcher mice did not significantly change over the 6-day testing period. Fingolimod-treated wildtype animals show significant changes in two locomotor parameters tested (distance travelled and average speed) when compared to healthy controls at PND28 and PND30.

3. Discussion

3.1. Summary of findings

Krabbe's disease (KD) is a rare neurodegenerative disorder characterised by severe demyelination and oligodendrocyte death (**Davenport et al. 2011**). Based on the findings of our earlier study that showed promising effects of fingolimod when administered to twitcher mice in a therapeutic manner (**Béchet et al 2020**), here we investigated whether prophylactic treatment could provide enhanced efficacy, before signs of clinical and underlying biochemical abnormalities. We administered vehicle (water) or fingolimod orally at a concentration of 1mg/kg/day, starting at postnatal day 5. Pathology of the cerebellum showed that early administration of fingolimod in twitcher mice reduced myelin debris, microglia reactivity and neuronal damage, while not regulating directly the levels of myelin or altering dysregulated astrocytes. The early treatment of twitcher mice with fingolimod, while not showing efficacy on weight or locomotor assessments, was found to reduce twitching and immobility and also increase lifespan, in manner comparable to treatment at post-weaning.

3.2. Fingolimod clears myelin debris in twitcher mice

Within the last decade, ample research has shown that the effects of fingolimod reach beyond that of immunomodulation and directly act within the CNS (**Dev et al. 2008, Miron et al. 2010**). Indeed, fingolimod induces process extension, enhances cell survival and stimulates cell maturation *in vitro* and *ex vivo* (**Coelho et al. 2007, Miron et al. 2008, O'Sullivan and Dev 2015**). When moving to *in vivo* studies, the fingolimod-mediated beneficial effects on myelin repair appear to be specific to particular animal models. For example, fingolimod failed to induce remyelination following acute and chronic cuprizone exposure (**Hu et al. 2011, Kim et al. 2011, Slowik et al. 2015**). It also did not significantly contribute to myelin repair following focal demyelination induced by lysolecithin injection in rat spinal cord (**Hu et al. 2011**). Here we found that the markers of myelin, MBP and MOG, did not increase with prophylactic treatment of fingolimod in neonatal twitcher mice. At first sight, this result appears somewhat surprising considering the beneficial effects of fingolimod when administered at post-weaning (**Béchet et al. 2020**). On reflection, however, this discrepancy may involve a differential developmental expression of S1P receptor subtypes and the opposing roles they play on immature and mature oligodendrocyte cell lineage during development, as previously reported (**Jaillard et al. 2005, Jung et al. 2007, Miron et al. 2008**). Moreover S1P and its receptors are integral for proper

neural and vascular development during embryonic stages (**Mizugishi et al. 2005**), which may have subtle differences in mice treated with fingolimod very early versus late treatment. Alternative endogenous S1P receptor signalling and the functional antagonism by fingolimod at early stages of development versus post-weaning, may therefore explain the differing effects we observed in these fingolimod-treated twitcher mice.

3.3. The neuroprotective effects of fingolimod in twitcher neonates

The neuroprotective spectrum of fingolimod has been illustrated in various *in vivo* and *in vitro* studies (**Cipriani et al. 2015, Slowik et al. 2015**). While remyelination in itself is considered neuroprotective, it is not an absolute necessity for neuroprotection. The relationship between axonal damage and remyelination is complex, and may occur independently (**Manrique-Hoyos et al. 2012, Slowik et al. 2015**). Here, we show fingolimod to improve axonal damage independent of myelination levels. Such results support the hypothesis that fingolimod preserves CNS integrity by direct interaction with neurons. Indeed, neurons are known to express S1P receptors (**Choi and Chun 2013**), and could thus be a direct target of fingolimod. Studies have supported S1P₁ receptor mediated neuroprotection by fingolimod *in vitro* in cortical primary and organotypic slice cultures exposed to NMDA excitotoxicity, as well as in animal models of Parkinson's disease and kainic acid neurodegeneration (**Cipriani et al. 2015, Zhao et al. 2016**). In addition to neuroprotection via direct S1P receptor modulation on neurons, the observed dampened axonal damage in our study could also be linked to the concurrent decrease in myelin debris, as measured by dMBP immunofluorescence. It is well established that myelin debris accumulation inhibits axonal regeneration and remyelination (**Kotter et al. 2006, Lampron et al. 2015**). Our results show that white matter tracts in the cerebellum of vehicle-treated twitcher mice are burdened with significant levels of myelin debris, compared to wildtype animals. This debris accumulation was alleviated with fingolimod treatment in twitcher mice and may also in part assist in the protection against axonal damage.

3.4. Fingolimod reduces microglia activation in twitcher mice

The psychosine hypothesis introduced in 1972 attributes the pathogenesis of KD to supraphysiological agglomerations of psychosine (**Suzuki 1998**). While this has been the predominant explanation, emerging evidence also ascribes early demise in KD to neuroinflammatory events (**Nicaise et al. 2016**). Microglial activation and globoid cell formation, alongside astrocyte activation, precedes demyelinating processes, thus attributing

an important and potentially exacerbating role to the innate immune system (**Nicaise et al. 2016, Potter and Petryniak 2016**). Here, we show that twitcher mice exhibit a substantive increase in microglial volume and a morphological change from a ramified to an amoeboid state accumulating in white matter tracts, which mirrors results from numerous previous experiments (**Ohno et al. 1993, Mohri et al. 2006, Kondo et al. 2011, Snook et al. 2014**). Fingolimod administration partially reversed the reactive morphology of microglia in our study, which was reflected by a decrease in volume of Iba1-positive cells. This finding agrees with *ex vivo* results from organotypic slice cultures, reporting a decrease in phagocytosing microglia with fingolimod accompanied by an increase of microglia numbers (**Miron et al. 2010**). Further *in vivo* findings have shown similar effects where fingolimod reduces neurodegeneration as well as microglial activation in a kainic acid-induced model of neuronal death and inflammation (**Cipriani et al. 2015**).

3.5. Early administration of fingolimod on astrocytes in twitcher mice

In twitcher mice, astrocytes undergo morphological changes associated with activation, with evident astrogliosis in the hindbrain and cerebellum of twitcher mice as early as 3 weeks of age (**Snook et al. 2014**). Here, we show that vimentin fluorescence intensity was significantly increased in twitcher mice, and GFAP expression analysed by immunoblotting saw similar increases in both untreated and treated twitcher mice. These results are in accordance with previous studies investigating immune and inflammatory events in KD, describing extensive astrogliosis surrounding white matter tracts in twitcher mice (**Lin et al. 2005, Snook et al. 2014, Hawkins-Salsbury et al. 2015**). *In vitro*, fingolimod regulates a number of signalling pathways in both rodent and human astrocytes, where S1P receptor drugs attenuate psychosine-mediated apoptosis of astrocytes (**O'Sullivan and Dev 2015**). Increased GFAP immunoreactivity is linked to demyelination in organotypic slice culture where fingolimod further increases in GFAP expression when was applied to cultures (**Miron et al. 2010**). Here, we found no changes in astrocyte levels with fingolimod treatment. Furthermore, neonatal administration of fingolimod did not modulate astrocyte reactivity in areas of demyelination. Of interest, we did find vimentin positive astrocytes to be relocated to white matter tracts in twitcher mice and this relocation was reduced by fingolimod, in line with previous studies showing the regulation of astrocyte migration by this drug (**Mullershausen et al. 2007**). There may be multiple reasons for differences in effects of fingolimod on astrocytes in twitcher mice when initiated early at PND5 versus PND21. These may include, the general role of astrocytes in neonatal versus young adult mouse brains, the differential expression, signalling and roles of S1P receptors in astrocytes

during development, and the effects of an activation, functional antagonism or persistent signalling of S1P receptors by fingolimod at a particular age (**Mullershausen et al. 2009**).

3.6.Limited peripheral effects of fingolimod in twitcher animals

While psychosine exerts its cytotoxic effects within the brain (**Contreras et al. 2008, Giri et al. 2008, Cantuti-Castelvetri et al. 2012, Misslin et al. 2017**), the pathology of KD also extends to peripheral organs with increased psychosine accumulation (**Whitfield et al. 2001, Li et al. 2019**). In sciatic nerves of twitcher mice, peripheral neuropathy show abnormalities in myelin as early as 1 week postnatal (**White et al. 2009, Smith et al. 2011**). In agreement, we found sciatic nerves showed severe reductions of myelin and Schwann cell markers, as well as increased expression of GFAP. However, our results did not reveal any beneficial effects of fingolimod. While fingolimod has previously been shown to promote peripheral axon regeneration and remyelination following sciatic nerve crush injury, our findings sit in line with no fingolimod-mediated effect on myelination in neuronal and Schwann cell co-cultures (**Kohne et al. 2012**). In closing, while we had hoped for a more promising effect of fingolimod on lifespan, our results admit a defeat to the well-known multi-organ involvement of leukodystrophies, where fingolimod may provide only partial efficacy. While disappointing, these findings point to the development of treatments for KD that can target multiple mechanisms, that include reduction of the levels of psychosine, while also reducing its toxicity in peripheral organs and the brain. We consider KD as a multimodal illness that will require targeting of multiple systems and perhaps a set of drugs that may be used in a combinatorial manner to target the various mechanisms of this illness.

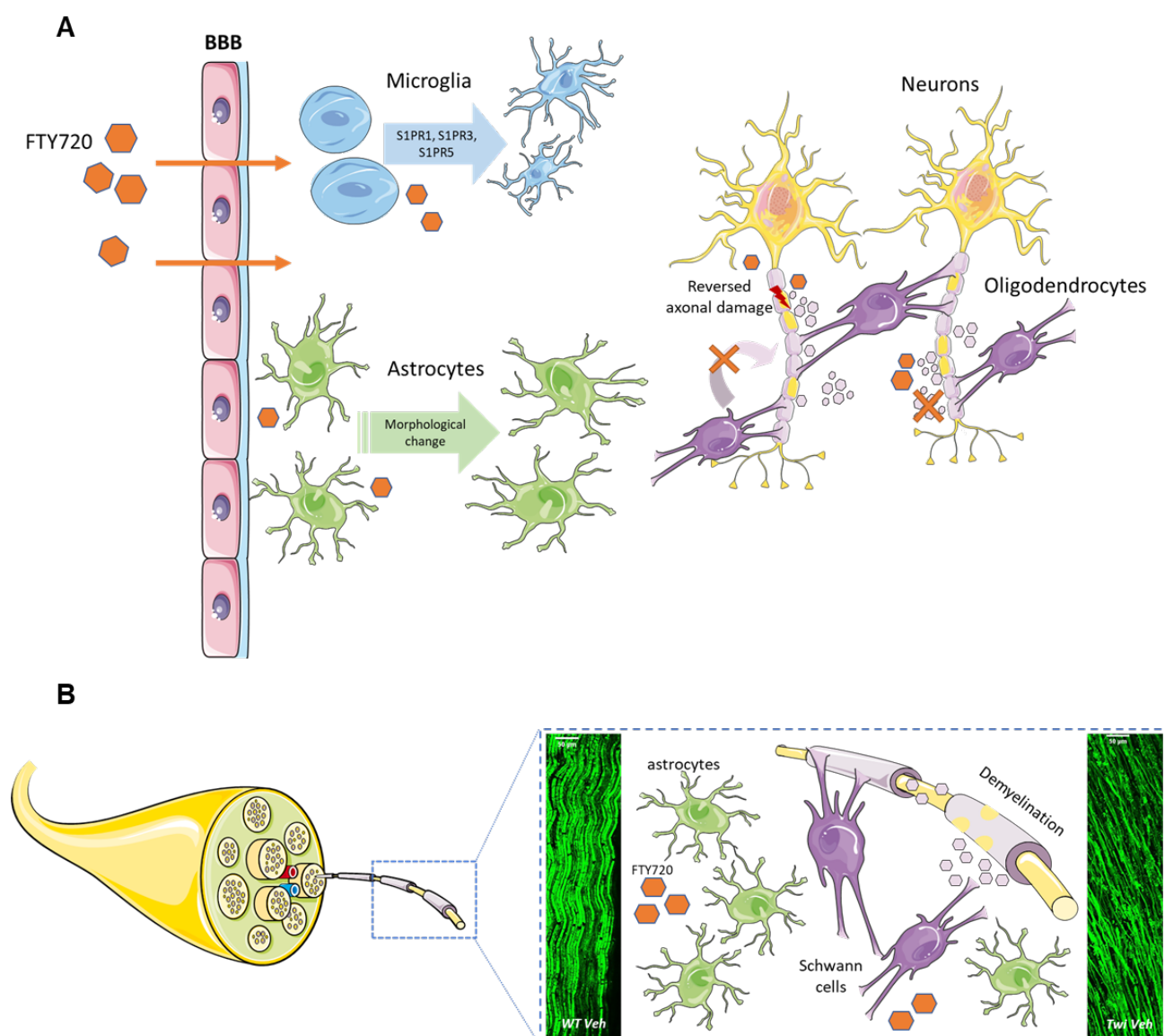


Figure 4.9 Summary figure of histological findings in twitcher mice following neonatal fingolimod administration

(A) Fingolimod administered at PND5 was able to cross the Blood Brain Barrier (BBB) and act on CNS-resident cells. Similar as with later fingolimod administration, we observed a change in microglial morphology, mediated by S1PR1, 3 and/or 5 modulation. Fibrous astrocyte numbers are elevated in white matter tracts of twitcher mice and were not altered with fingolimod administration. However, qualitative analysis suggests a change in their morphology. Interestingly, neonatal administration of fingolimod resulted in reduced myelin debris accumulation and dampened axonal damage, without however increasing overall myelin levels. **(B)** The severe demyelination of sciatic nerves and surrounding inflammation and myelin debris was not decreased by fingolimod treatment.

Chapter 5 – The dual S1PR_{1/5} agonist BAF312 (Siponimod) does
not attenuate demyelination in twitcher mice

Chapter Aims

The immunosuppressive mechanism of action of siponimod could provide a benefit in KD by preventing inflammation in response to globoid cell accumulation, and by attenuating reactivity of microglia and astrocytes. This, in turn, could have a beneficial effect on myelination and myelin debris accumulation. Our lab has previously shown that the dual S1PR_{1/5} compound siponimod was able to attenuate LPC- and psychosine-induced demyelination in organotypic slice cultures (**O'Sullivan et al. 2016**). Additionally, siponimod was shown to induce levels of pERK and pAKT in human and mouse astrocytes (**O'Sullivan et al. 2016**). Such in vitro findings suggest that siponimod may regulate myelination in inflammatory models of demyelination. This prompted the current study, investigating the role of the S1PR₃ sparing drug siponimod in twitcher mice, the murine model of KD.

Specific aims of this chapter included:

- To examine the relative importance of S1PR₁, S1PR₃ and S1PR₅ modulation in demyelination and inflammation, using a murine model of KD.
- To determine the effect of S1PR_{1/5} modulation on markers of myelination using siponimod and its potential in attenuating demyelination in the twitcher mouse model.
- To study the efficacy of siponimod in reducing neuroinflammation, as measured by glial cell reactivity.
- To examine the influence of siponimod on the infiltration of globoid cells into the white matter parenchyma of twitcher mice.
- To assess behavioural outcomes including lifespan, ataxia, immobility and weight loss, of siponimod treated twitcher mice compared to vehicle (water)-treated twitcher and wildtype animals.

Abstract

The therapeutic value of Sphingosine 1-Phosphate receptor modulation was highlighted by the success of fingolimod (FTY720, Gilenya) in treating relapsing remitting MS. This clinical success was followed by efforts to develop more selective agonists and antagonists towards S1PR subtypes, particularly S1PR_{1/5}. Avoiding activation of S1PR₃ was of particular interest as that receptor subtype has been linked to transitory bradycardia and vasculitis. Furthermore, favourable properties associated with S1PR_{1/5} made these attractive receptor subtypes to target in neurodegeneration. Here, we investigated the effects of selective S1PR_{1/5} modulation with the S1PR₃ sparing compound BAF312 (Siponimod, Mayzent) in twitcher mice, the murine model of Krabbe's disease (KD). Behavioural findings emerging from this study suggest that selective modulation of S1PR_{1/5} with siponimod does not significantly reduce phenotypic abnormalities or increase life span in twitcher mice. Myelination levels, inflammatory status and axonal injury were analysed using immunohistochemistry. Here, we found myelination levels to be unchanged in siponimod treated twitcher mice, with no significant changes in myelin debris accumulation. However, fibrous astrocytes that are increased in white matter tracts of untreated twitcher mice were decreased with siponimod treatment. Similarly, we noted a significant decrease in Iba1-positive cells in the cerebellum of twitcher mice. No significant changes in peripheral organ (spleen, kidney) size was observed. Together, such results insinuate that selective S1PR_{1/5} modulation is sufficient to modulate microglia and astrocyte activation. However, the lack of remyelination observed with siponimod treatment suggests that S1PR₃ modulation may playing a key role in remyelination.

Keywords: BAF312; Siponimod; Globoid cell leukodystrophy; Krabbe's disease; myelination; neuroinflammation

1. Introduction

Over the past decade, the family of sphingosine 1-phosphate (S1P) receptors have been rapidly gaining attention as important mediators of a variety of vital cellular processes, including cell proliferation, migration, differentiation, angiogenesis, homeostasis and inflammation and immunity (**Brinkmann, 2007; Dev et al. 2008**). These G-protein coupled receptors are known targets for the drug fingolimod (FTY720, Gilenya®), an oral therapy used for treatment of relapsing remitting multiple sclerosis (RRMS). The development of fingolimod and its demonstrated clinical efficacy in RRMS has elicited ongoing efforts to develop more selective agonists and antagonists targeting the S1P axis (**Kunkel et al. 2013, O'Sullivan and Dev 2017**). Siponimod (BAF312, Mayzent®) is an orally administered drug for the treatment of secondary progressive MS (**Pan et al. 2013**). It was developed by Novartis Pharmaceuticals, and licensed by the European Medicines Agency in January 2020 (**Al-Salama 2019**). Siponimod was developed as an S1P receptor modulator, with selective affinity for S1PR₁ and S1PR₅ subtypes. The compound thereby avoids interaction with S1PR₃, a receptor associated with bradycardia and vasoconstriction, one of the side effects observed with fingolimod. Of note, reports from clinical trials show that siponimod still induced bradycardia, unless mitigated using dose-titration schemes, or under polypharmacy with propranolol (**Legangneux et al. 2013, Biswal et al. 2015, Kappos et al. 2018**). Currently, siponimod is being investigated in clinical trials of various phases in patients with hepatic impairment (NCT01565902), polymyositis (NCT01801917), dermatomyositis (NCT02029274) or haemorrhagic stroke (NCT03338998).

KD is a fatal rare inherited lipid storage disorder predominantly affecting infants. This illness is caused by mutations in the *galc* gene encoding the enzyme galactosylceramidase (GALC). Enzymatic dysfunctions lead to the cytotoxic build-up of the galactolipid psychosine, which, in turn, induces oligodendrocyte apoptosis and severe demyelination (**Graziano and Cardile 2015**). We have previously shown fingolimod to mediate remyelinating effects via S1PR₁, S1PR₃ and S1PR₅ modulation in twitcher mice, a murine model of KD (**Béchet et al. 2020**). Oligodendrocytes preferentially express S1PR₅, the activation of which can mediate process retraction in immature oligodendrocytes and cell survival in mature cells (**Jaillard et al. 2005**). Remyelination via S1P receptors has thus mainly been attributed to S1PR₅ and/or S1PR₁ modulation (**Miron et al. 2010, Sheridan and Dev 2012**). However, Miron and colleagues (**2010**) have also implicated S1PR₃-mediated signalling in remyelinating processes. Expression patterns of S1PRs vary depending on cell type, developmental stage and physiopathological condition (**Blaho and Hla 2014**). For instance, astrocytes have been shown to express S1PR₁ and S1PR₃

subtypes, with an increasing expression of S1PR₅ upon exposure to growth factors (**Rao et al. 2004**). Additionally, S1PR₁ and S1PR₃ are increased in reactive astrocytes (**Fischer et al. 2011**).

The current study aimed to examine the relative importance of S1PR₁, S1PR₃ and S1PR₅ modulation in demyelination and inflammation, using the selective S1PR_{1/5} agonist siponimod in twitcher mice. Our lab has previously shown siponimod to induce S1PR₁ internalisation in astrocyte cultures and to model levels of ERK and AKT phosphorylation as well as calcium signalling pathways (**O'Sullivan et al. 2016**). Importantly, siponimod was able to reduce levels of IL-6 and attenuate demyelination in organotypic slice cultures (**O'Sullivan et al. 2016**). These results prompted the current investigation of this dual S1PR_{1/5} agonist as a therapeutic intervention in KD. Additionally, specific S1PR_{1/5} modulation with the S1PR₃ sparing compound will determine the importance of S1PR₃ subtype in remyelination and inflammation. Our outcome measures included both behavioural phenotypic and histological assessment of myelination status, myelin debris accumulation, microglial and astrocyte activation, and axonal damage.

2. Results

2.1. The dual modulation of S1P_{1/5} receptors reduces levels of myelin in young adult mice.

The dual S1PR_{1/5} agonist BAF312 (siponimod) attenuates demyelination in organotypic cerebellar slice cultures treated with psychosine or lysophosphatidylcholine (LPC) (O'Sullivan et al., 2016). To investigate these effects *in vivo*, we administered siponimod orally at a concentration of 1mg/kg/day to both twitcher and wildtype animals. The myelin status was measured by examining the expression levels of MOG and MBP. In an effort to ethically minimise the number of severe twitcher mice used, we added animal numbers to the vehicle-treated groups reported in our previous study (Béchet et al. 2020). Increasing the group size of both vehicle-treated wildtype and twitcher mice, strengthened statistical significance of our original data showing that MOG and MBP immunostaining in vehicle-treated twitcher mice was significantly lower compared to wild-type mice (MOG: WT^{Veh} 100±7.4 vs Twi^{Veh} 56.3±3.5, ****p<0.0001, df=34, n=13, previously n=8) (MBP: WT^{Veh} 100±10.3 vs Twi^{Veh} 54.5±4.3, ***p=0.0002, df=33, n=13, previously n=10) (Figure 5.1A). While fingolimod had no effect on levels of MOG (Béchet et al. 2020), the administration of siponimod reduced significantly the expression level of MOG in wildtype mice (WT^{BAF} 62.02±9.3, **p=0.0065, df=34, n=6), with no rescue in the levels of MOG in siponimod-treated twitcher mice compared to vehicle treatment (Twi^{BAF} 59.7±10.5, ^{ns}p=0.9886, df=34, n=6) (Figure 5.1A). Siponimod also had no significant effects on the expression levels of MBP in wildtype mice (WT^{BAF} 75.2±8.3, ^{ns}p=0.1654, df=33, n=5) or twitcher mice (Twi^{BAF} 71.8±11.4, ^{ns}p=0.6568, df=33, n=5), compared to vehicle treated animals (Figure 5.1A), and in contrast to fingolimod, which restored the levels of MBP in twitcher mice (Bechet et al 2020). The ability to clear myelin debris is a critical component of the remyelination response, a phenomenon known to be impaired in demyelinating diseases (Lampron et al. 2015, Lloyd and Miron 2019). Reanalysis, with reselected ROI's, of the vehicle-treated wildtype and twitcher mice data taken from our previous study (Béchet et al. 2020), showed myelin debris in vehicle treated twitcher mice was increased, with the presence of extensive dMBP positive clusters in twitcher mice compared to wildtype mice (WT^{veh} 100±6.9 vs Twi^{veh} 156.2±9.96, ***p=0.0005, df=12, n=4) (Figure 5.1B). Similar to fingolimod (Béchet et al. 2020), this debris accumulation was not altered by siponimod in wildtype (WT^{BAF} 82.97±6.3, p=0.3563, df=12, n=4) or twitcher mice (Twi^{BAF} 178.3±4.2, p=0.1714, df=12, n=4), compared to vehicle treatment (Figure 5.1B). These data suggest that siponimod alters basal levels of myelin, without leading to myelin debris, when given to young adult mice starting at PND21. The

observed differential effects of fingolimod and siponimod may be due to their specific pharmacology and the roles individual S1PR subtypes play in myelination during early development.

2.2. Astrocyte reactivity in twitcher mice is not altered by siponimod.

The morphology of astrocytes in twitcher mice display a higher number of processes, longer branches and increased diameter, generally preceded by microglial and macrophage activation (Snook et al. 2014). Siponimod and fingolimod promote pERK, pAKT and calcium signalling in mouse and human astrocytes, where S1PR₁ and S1PR₃ play subtly differing roles (O'Sullivan et al. 2016). *In vitro*, psychosine induces astrocyte apoptosis in a concentration- and time-dependent manner, which is rescued by fingolimod (O'Sullivan and Dev 2015). In twitcher mice, the expression of the astrocytic marker vimentin, but not GFAP, is reduced in the Bergmann glial cells in the molecular layer (ML) of the cerebellum with a concomitant increase in fibrous astrocytes located in the white matter (WM), that is altered by fingolimod (Béchet et al. 2020). Here, we examined the expression of vimentin and GFAP in vehicle- and siponimod-treated wildtype and twitcher mice. For vehicle-treated wildtype and twitcher mice groups, we again added animal numbers to the existing results reported in our previous study (Béchet et al. 2020). In the ML of the cerebellum, the expression of vimentin decreased significantly of twitcher mice (WT^{Veh} 100±10.4 vs Twi^{Veh} 55.8±4.7, **p=0.0088, df=22, n=7-9, previously n=5) (Figure 5.2A). The administration of siponimod had no statistically significant effect on the expression of vimentin in the ML of the cerebellum in wildtype (WT^{BAF} 101.5±8.6, p=0.9860, df=22, n=5) or twitcher mice (Twi^{BAF} 84.6±8.4, p=0.1730, df=22, n=5), compared to their vehicle-treated counterpart groups. The expression of vimentin increased significantly in the WM of twitcher mice (WT^{Veh} 100±11.6 vs Twi^{Veh} 219.3±20.8, ****p<0.0001, df=24, n=9-10, previously n=5-6). In the WM of the cerebellum, siponimod reduced significantly the expression of vimentin in twitcher mice (Twi^{BAF} 132.5±9.9, *p=0.0104, df=25, n=5), with no effect in wildtype mice (WT^{BAF} 124.9±10.9, p=0.7681, df=25, n=5) (Figure 5.2A). Addition of data to our previous study (Béchet et al. 2020), also statistically supported that GFAP immunofluorescence did not change across groups, where siponimod had no effect (Figure 5.2B), similar to fingolimod (Béchet et al. 2020). Western blotting using homogenised cerebellum, showed increased expression of total GFAP with no effect of siponimod (Figure 5.2B), in agreement with our previous study and similar to fingolimod (Béchet et al. 2020). Overall, these data confirm altered astrocyte function in twitcher mice and suggest that fingolimod and siponimod regulate differentially astrocytes, which may be dependent on their S1PR₁ and S1PR₃ pharmacology and altered signalling in these cells (O'Sullivan et al. 2016, Béchet et al. 2020).

2.3. Siponimod significantly reduces Iba1 immunoreactivity in twitcher mice

The protein Iba1 is expressed by microglia and macrophages and plays a role in regulating activation of microglia (**Mori et al. 2000**). Microglia activation is strongly associated with inflammation and progression of KD inflammation, with recent findings attributing early demise observed in KD in part to neuroinflammation rather than sole oligodendrocyte loss and demyelination (**Nicaise et al. 2016, Potter and Petryniak 2016**). Findings suggest also that microglial activation promotes activation of neighbouring astrocytes, contributing to neuroinflammation and demyelinating pathology of KD (**Snook et al. 2014**). To investigate the role of siponimod in attenuating microglia response in twitcher mice, we stained cerebellar slices with an antibody against Iba1. Results confirmed our earlier study (**Béchet et al. 2020**), with an expected increase in Iba1-positive cells surrounding the white matter parenchyma in the cerebellum of vehicle-treated twitcher mice ($WT^{Veh} 100 \pm 19$ vs $Tw^{Veh} 280.6 \pm 26.1$, *** $p=0.0001$, $df=12$, $n=4$) (**Figure 5.3A**). Similar to fingolimod, siponimod significantly reduced the number of Iba1-positive cells in twitcher mice, when compared to vehicle-treated twitcher mice ($Tw^{Veh} 280.6 \pm 26.1$ vs $Tw^{BAF} 180.3 \pm 20.8$, * $p=0.016$, $df=12$, $n=4$) (**Figure 5.3A**). The pathophysiology of KD is characterised by the presence of multinucleated macrophages termed globoid cells, a particular feature and histological hallmark of this leukodystrophy in both humans and mice (**Suzuki 2003**). Phagocytosis of galactosylceramide, a cerebroside native to the myelin sheath, leads to the morphological transformation of macrophages and/or microglia into Periodic-Acid Schiff (PAS) positive globoid cells (**Graziano and Cardile 2015**). To investigate the potential effects of siponimod on the presence of globoid cells, cerebellar slices of twitcher mice and wildtype littermates were stained with Luxol Fast Blue (LFB) and PAS, to visualise myelin and globoid cells, respectively. As expected, we observed reduced levels of LFB and a significant increase in PAS positive cells surrounding myelinated areas in vehicle treated twitcher mice when compared to healthy controls ($WT^{Veh} 0.92 \pm 0.6$ vs $Tw^{Veh} 42.44 \pm 4.5$, **** $p<0.0001$, $df=20$, $n=6$) (**Figure 5.3B**). Siponimod treatment did not significantly alter PAS-positive cell numbers in either wildtype animals ($WT^{BAF} 0.75 \pm 0.4$, $^{ns}p>0.9999$, $df=20$, $n=6$) or twitcher mice ($Tw^{BAF} 30.62 \pm 6.4$, $^{ns}p>0.05$, $df=20$, $n=6$), when compared to control animals (**Figure 5.3B**).

2.4. Increased expression levels of neuronal SMI32 in twitcher mice

Psychosine induces dephosphorylation of neurofilament subunits (**Cantuti-Castelvetri et al. 2012**), which likely contributes to neurological phenotypes observed in the twitcher mice, including tremor, ataxia and progressive muscle atrophy. While these symptoms have long been linked to ongoing demyelination and consequent axonal damage, reports suggest axonal thinning and dephosphorylation can occur independent of myelination state in the absence of substantial demyelination, as early as PND12 (**Castelvetri et al. 2011, Cantuti-Castelvetri et al. 2012**). To investigate the phosphorylation state of neurons in our twitcher mice, and the potential of siponimod in attenuating axonal damage, we stained cerebellar slices against neurofilament heavy (NFH) as well as SMI32, a non-phosphorylated NFH epitope, as we have previously reported (**Béchet et al. 2020**). For vehicle-treated wildtype and twitcher groups, we built on the animal numbers of our previous study (**Béchet et al. 2020**). In that study we observed a level of variance across animals, and particularly in twitcher mice, that consequently did not give a quantitative statistical difference between wildtype and twitcher mice, despite showing a qualitative increase in the expression of SMI32 in twitcher animals. We again observed high variability of SMI32 immunofluorescence in twitcher mice, however by doubling the group size of these animals in this current study, a statistically significant increase was reached in the expression of SMI-32 in WM tracts of twitcher mice, when compared to vehicle-treated wildtype littermates (WT^{Veh} 100±10.6 vs Twi^{Veh} 174.33±13.3, **p=0.0013, df=36, n=14-15, *previously n=6-7*) (**Figure 5.4A,B**). The expression levels of SMI32 in WM was not altered by siponimod in either wildtype animals (WT^{BAF} 160.9±20.3, ^{ns}p=0.0948, df=36, n=5) or in twitcher mice (Twi^{BAF} 193.7±27.6, ^{ns}p=0.8496, df=36, n=6), compared to vehicle-treated animal counterparts (**Figure 5.4A,B**), similar to the effect of fingolimod (**Béchet et al. 2020**). No significant differences were observed across groups in the expression of SMI32 in the ML (WT^{Veh} 100±10.5, Twi^{Veh} 133.2±14.8, WT^{BAF} 144.6±16.2, Twi^{BAF} 154.9±26.8, n=5-12) (**Figure 5.4A,C**) or in NFH immunofluorescence (WT^{Veh} 100±14.5, Twi^{Veh} 80.4±7.3, WT^{BAF} 84.9±6.95, Twi^{BAF} 87.27±11.5, n=5) (**Figure 4A,C**). These data are in agreement, in best part, with studies showing that twitcher mice have neuronal damage, and this is not robustly attenuated by drugs such as fingolimod or siponimod.

2.5. Siponimod delays weight loss but does not improve twitching and immobility scores in twitcher mice.

The modulation of S1P receptors with fingolimod significantly improves mobility, twitching severity, body weight, and prolongs life span (**Béchet et al. 2020**). Here, the administration of siponimod in twitcher mice delayed weight loss at an early age, although this did not affect lifespan. Interestingly, the administration of siponimod resulted in increased twitching severity, which were noted at PND30 (Tw^{Veh} vs Tw^{BAF} , **** $p < 0.0001$), PND32 (Tw^{Veh} vs Tw^{BAF} , * $p = 0.0480$) and PND37 (Tw^{Veh} vs Tw^{BAF} , *** $p > 0.0001$) (**Figure 5.5A**). Similarly, hind limb paralysis worsened with siponimod treatment between postnatal days 30-32 and days 36-37, with somewhat relapsing trends in-between and converging back to those of vehicle treated animals at PND38 (**Figure 5.5B**). Siponimod positively affected body weight, with a significant improvement at an early stage of life between PND23 and PND29, and thereafter weight gain plateaued and converged towards that of vehicle treated animals at PND30 (**Figure 5.5C**). The Kaplan-Meier survival curves shows no significant improvement with siponimod in the lifespan of twitcher mice versus vehicle treated littermates ($p = 0.0687$) (**Figure 5.5D**). We again noted a decrease in the size and weight of visceral organs such as spleen and kidney of twitcher mice, compared to healthy controls (Spleen: WT^{Veh} 96.8mg ± 4.3 vs Tw^{Veh} 30.4mg ± 4 , **** $p < 0.0001$, $df = 22$, $n = 6$; Kidney: WT^{Veh} 189.4mg ± 12.3 vs Tw^{Veh} 71.6mg ± 3.5 , **** $p < 0.0001$, $df = 22$, $n = 6$) (**Figure 5.5E-F**). The administration of siponimod modestly reduced the weight of spleen in wildtype mice (WT^{BAF} 81.8mg ± 3.7 , * $p = 0.0315$, $n = 8$, $df = 22$) but did not alter weight of spleen in twitcher mice (Tw^{BAF} 25.7mg ± 1.7 , $p = 0.8206$, $n = 4$, $df = 12$), compared to vehicle-treated animal counterparts (**Figure 5.5E**). Siponimod had no effect on the right kidney in wildtype (WT^{BAF} 171.9mg ± 9 , $p = 0.4407$, $df = 22$, $n = 6$) or twitcher mice (Tw^{BAF} 97.8mg ± 3.9 , $p = 0.1704$, $df = 22$, $n = 4$) (**Figure 5.5F**). These findings are similar to that of fingolimod, where siponimod treatment results in some rescue of CNS pathology and behavioural phenotypes, but not in peripheral organ damage and loss.

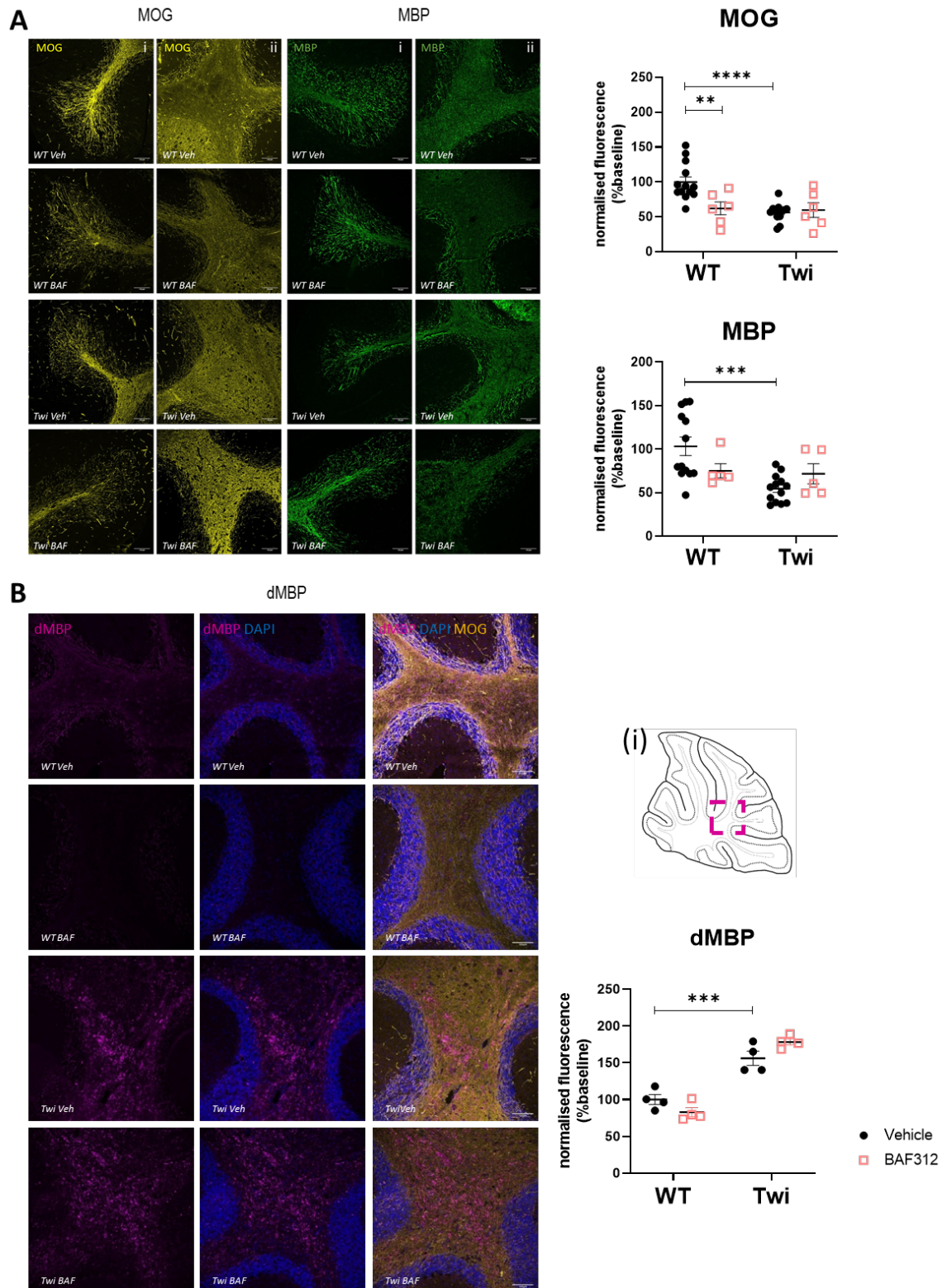


Figure 5.1 Siponimod does not improve myelination levels or myelin debris in cerebellar white matter of twitcher mice.

(A) Twitcher mice and wildtype littermates were treated with vehicle (water) or siponimod (1mg/kg/day) starting at PND21. Representative images show MOG (yellow) or MBP (green), in lobes (i) and white matter (ii) of the cerebellum across the four treatment groups. Quantification of MOG fluorescence showed a significant decrease in twitcher mice, compared to wildtype, with no significant effects of

siponimod. MBP fluorescence analysis showed no significant differences across groups. **(B)** Western blots and densitometric analyses of MOG and MBP immunoreactivity in cerebellar samples of vehicle- and BAF312-treated wildtype and twitcher mice. No significant changes were observed in MOG and MBP immunoreactivity across groups. Siponimod did not significantly increase MOG or MBP expression as measured by densitometry. **(C)** The extent of myelin debris in the cerebellar white matter was quantified by immunohistochemistry. Cerebellar slices were immunolabelled with a marker for degraded MBP (dMBP) (purple), and fluorescence intensity was measured using ImageJ. Images were taken in the region of the cerebellar white matter as depicted in the (i) cartoon diagram (dotted box). Using an Sp8 confocal, Z-stacks were taken at 0.68µm intervals and flattened to give the maximum projection immunofluorescence. Graph shows a significant increase in dMBP in twitcher mice compared to wildtype littermates, which was not significantly reduced with siponimod treatment. In all cases, confocal images taken at 20x magnification. Scale bar, 100µm. Data expressed as mean ±SEM, two-way ANOVA and Tukey's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Vehicle-treated animals (black circles) and siponimod-treated animals (orange boxes).

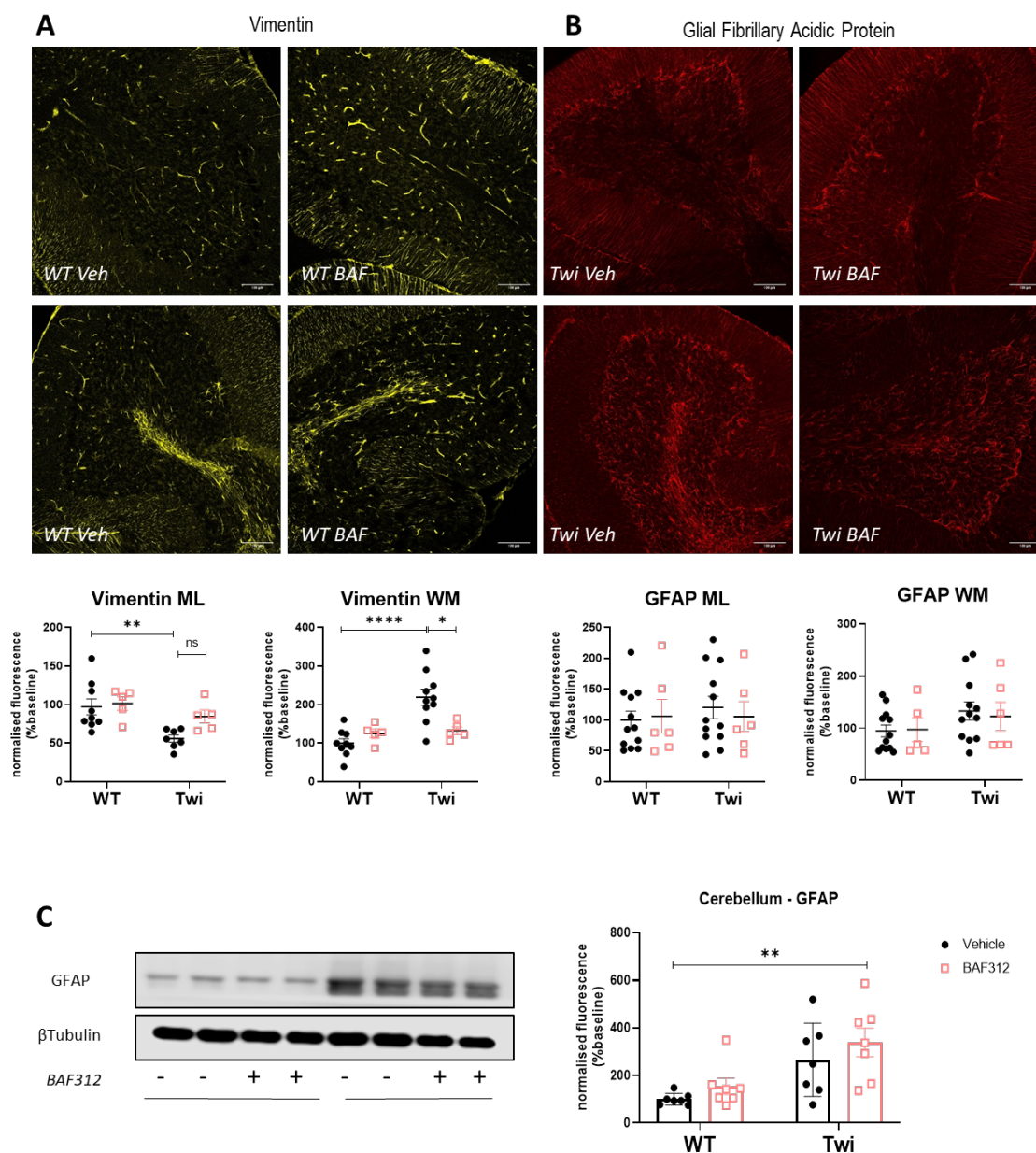


Figure 5.2 Vimentin but not GFAP expression is altered in the cerebellum of twitcher mice.

(A,B) Representative images of fibrous astrocytes in the white matter (WM) parenchyma and Bergmann glia in the molecular layer (ML) of the cerebellum. Fluorescence intensity was grouped into analysis of the molecular layer (ML) and the white matter (WM) tracts as depicted in the (i) cartoon and cresyl violet stain diagram (A) Analysis of vimentin (yellow) fluorescence in the ML shows no significant differences across the four treatment groups. Within the WM tracts, vimentin labelled cells were significantly increased in vehicle-treated twitcher mice when compared to wildtype littermates. This increase in the WM tracts was significantly decreased with siponimod in twitcher mice. (B) Analysis of GFAP (red) fluorescence in the ML and WM shows no significant differences across the four treatment groups. (C) Western blots and densitometric analyses of GFAP immunoreactivity in cerebellar samples of vehicle- and siponimod-treated wildtype and twitcher mice. GFAP immunoreactivity was significantly increased in both treated and untreated twitcher mice compared to wildtype, with no significant effects of siponimod. Confocal images taken at 20x. Scale bar, 100 μ m. Data expressed as mean $\pm$ SEM, two-way ANOVA and Tukey's post-hoc test (* p <0.05). Vehicle-treated animals (black circles) and siponimod-treated animals (orange boxes).

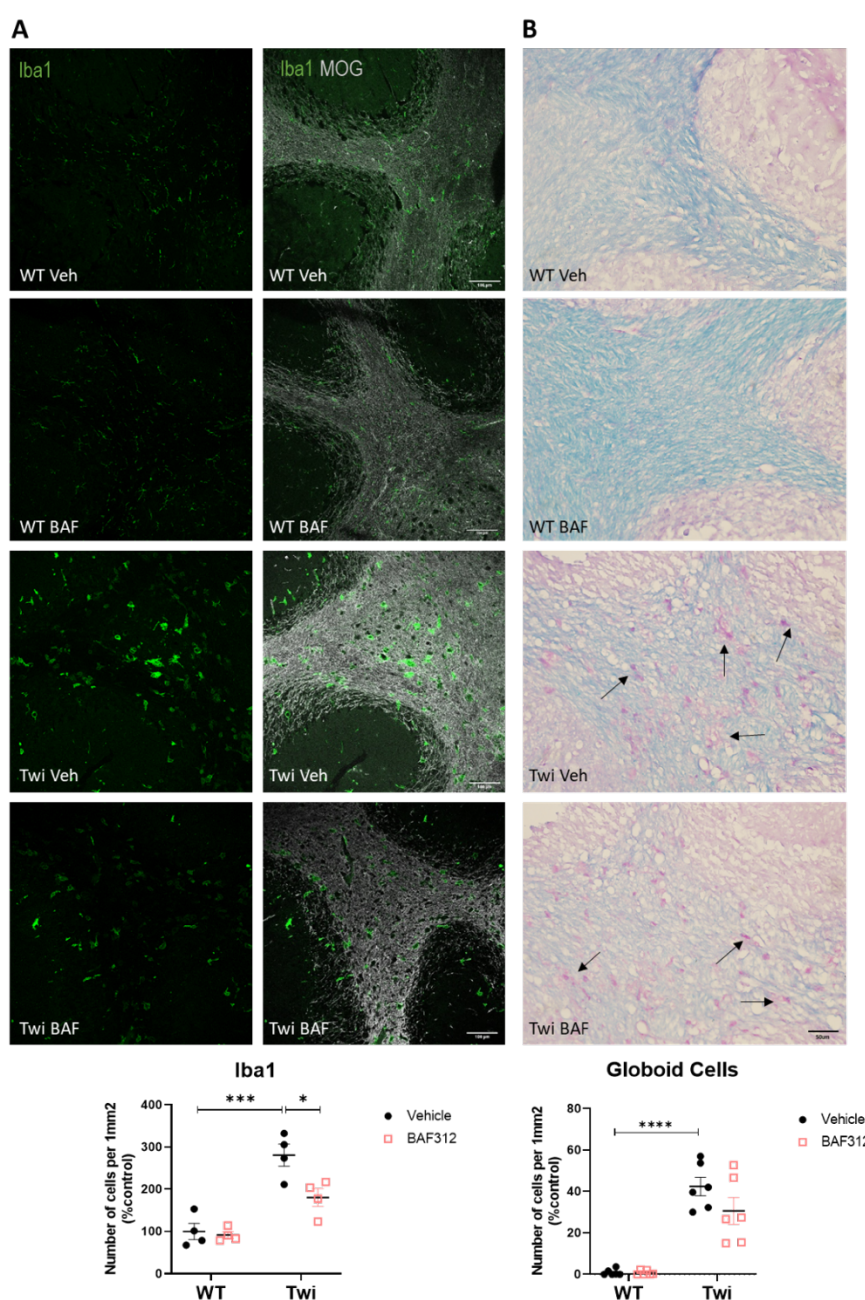


Figure 5.3 Sponimod significantly reduces Iba1 positive cells in the cerebellum of twitcher mice.

(A) Representative images of Iba1-positive microglia/macrophages (green) and MOG (grey) in the cerebellum of siponimod- and vehicle-treated wildtype and twitcher mice. (B) Graphs showing Iba1-positive cells per mm². There was a significant increase in Iba1-positive cells in the cerebellum of vehicle-treated twitcher mice, compared to wildtype animals. This was significantly reduced with siponimod treatment in twitcher mice (D) Graph showing PAS-positive cells per mm² across treatment groups. PAS-labelled globoid cell number was significantly increased in vehicle- and siponimod-treated twitcher animals, compared to wildtype mice. Siponimod did not attenuate this increase significantly in twitcher mice. (D) Representative images depicting PAS-positive globoid cells (purple) and myelin (Luxol Fast Blue/LFB – blue) in the cerebellum of Twitcher mice. All images taken at 20x magnification. Scale bar, 100 µm for confocal images. Scale bar 50 µm for PAS-LFB images. Data expressed as mean ± SEM, two-way ANOVA and Tukey's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

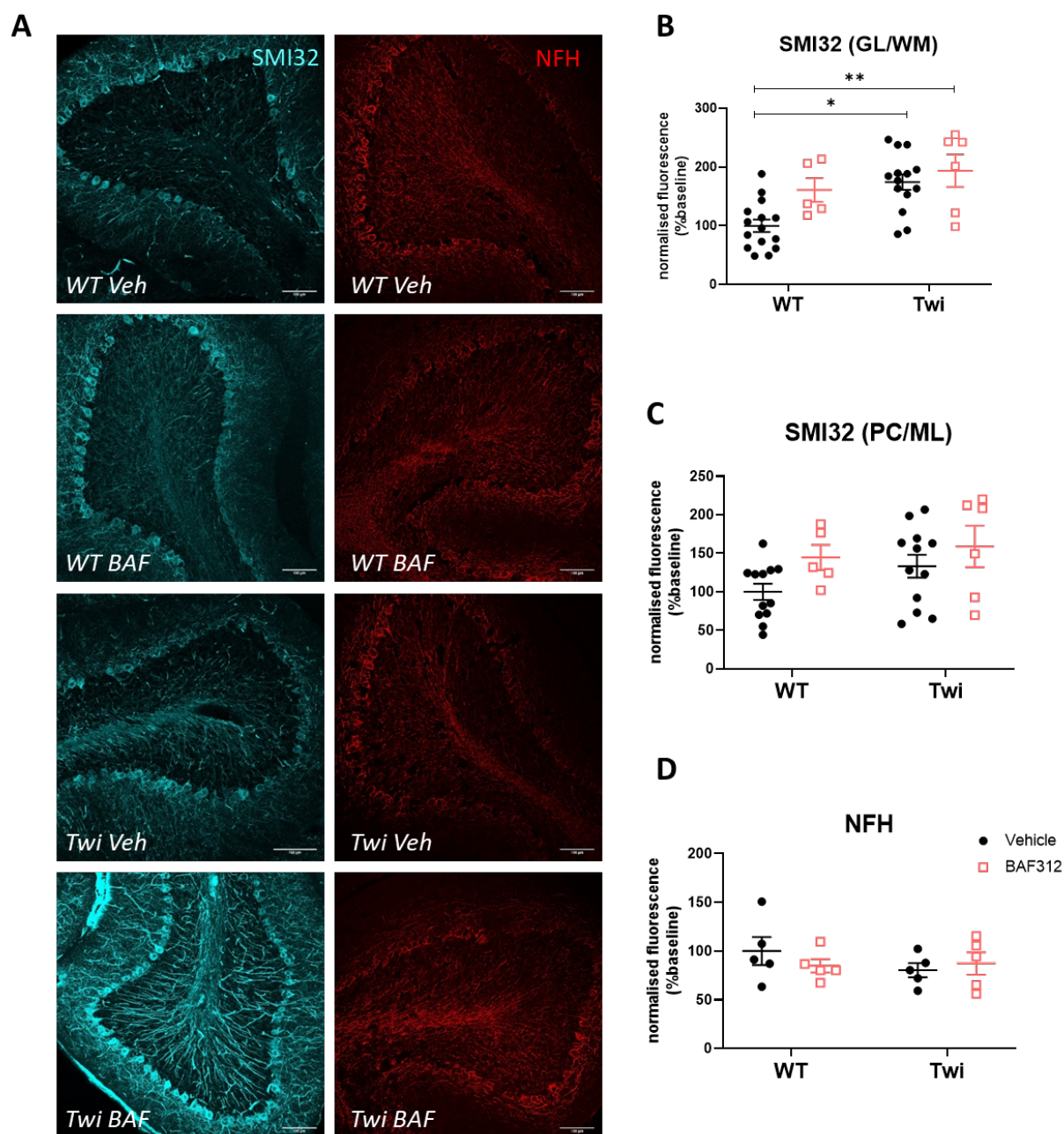


Figure 5.4 Siponimod does not alter the phosphorylation state of neurofilaments in white matter tracts of twitcher mice.

(A) Representative images of SMI-32 (cyan) and NFH (red) illustrating changes in phosphorylated and non-phosphorylated neurofilaments in the cerebellum slices across the four treatment groups. **(B)** No changes were observed in NFH. **(C)** SMI-32 fluorescence remained similar in Purkinje cell and molecular dendritic layers (PC/ML) with no significant changes across treatment groups. **(D)** SMI-32 expression was significantly increased in the cerebellar granular layer and white matter tracts (GL/WM) of vehicle- and siponimod treated twitcher mice. This was not reversed with siponimod treatment in twitcher mice. Confocal images taken at 20x magnification. Scale bar, 100µm. Data expressed as mean ±SEM, two-way ANOVA and Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

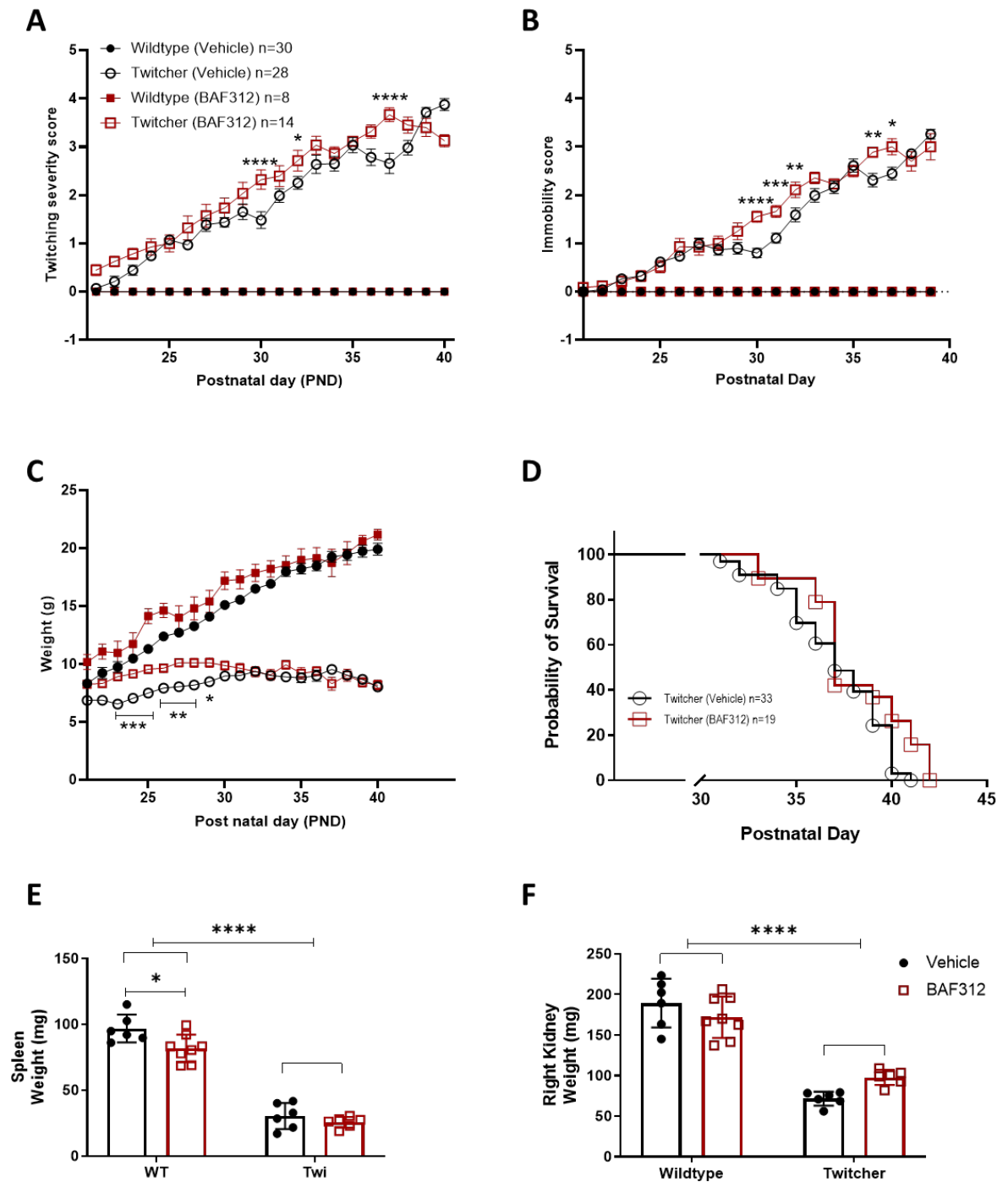


Figure 5.5 Siponimod does not improve survival in twitcher mice

(A) Twitching, (B) mobility and (C) weight were statistically assessed using a two-way ANOVA followed by a Tukey adjustment for multiple comparisons. The number of animals per group were: vehicle (n=13) and siponimod (n=8) treated wildtype mice and vehicle (n=13) and siponimod (n=14) treated twitcher mice. (D) The Kaplan-Meier survival curve illustrates changes with siponimod on lifespan of twitcher mice compared to vehicle treated twitcher mice. A log-rank test reveals no significant difference in survival distribution between vehicle- and siponimod treated twitcher mice. The statistical values are as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3. Discussion

3.1. Summary of findings

The rapid loss of oligodendrocytes and myelin is a predominant pathological feature of KD, and is responsible for slowed nerve conduction observed in Twitcher animals (**Davenport et al. 2011**). S1P receptor modulators have gained increasing popularity as therapeutics in neurodegenerative and demyelinating disorders (**Kunkel et al. 2013**). We have previously shown fingolimod to mediate protective effects via S1PR₁, S1PR₃ and S1PR₅ modulation in twitcher mice, a murine model of KD (**Béchet et al. 2020**). In order to further determine the differential role of these three S1P receptor subtypes, we administered the selective S1PR_{1/5} compound siponimod to twitcher mice, thus sparing S1PR₃ modulation.

Here, we found that siponimod was unable to attenuate demyelination and axonal damage observed in twitcher mice. Using a marker for degraded myelin, we also observed no changes in myelin debris accumulation with siponimod. We observed decreases in microglia positive cells, but no significant change in globoid cell numbers. Astrogliosis was partially decreased in white matter tracts of siponimod treated twitcher mice. These modest findings were reflected in a partially and sporadically improved locomotion and ataxia. Twitching, immobility and weight scores showed mild improvements but ultimately converged back towards similar scores of untreated twitcher mice. Siponimod treatment did not result in significant increases of life span compared to vehicle treated twitcher mice.

3.2. Siponimod does not attenuate demyelination in Twitcher mice

Siponimod was developed as an S1P receptor modulator, with selective affinity for S1PR₁ and S1PR₅ subtypes and a limited activity profile for S1PR₃ (**Pan et al. 2013**). Reasons for this, in most part, are to avoid potential side effects such as bradycardia and vasoconstriction, induced by S1PR₃ activation (**Legangneux et al. 2013**). Here, we used the S1PR₃ sparing drug siponimod to investigate the role of dual S1PR_{1/5} activation in remyelination and inflammation in twitcher mice. We found that neither myelination makers were significantly increased with siponimod in twitcher mice, when compared to vehicle-treated groups. Considering the promising histological findings we described earlier with fingolimod, this lack of remyelination with siponimod emphasises the importance of S1PR₃ activation in myelinating processes. Enhanced remyelination has previously been credited to fingolimod-mediated action on both S1PR₃ and

S1PR₅ (**Miron et al. 2010**). Using specific S1P receptor agonists and antagonists, Miron and colleagues (**2010**) showed that enhanced remyelination in organotypic slices was primarily mediated via S1PR_{3/5} signalling. Blocking that pathway using the antagonist suramin reversed the enhanced remyelination, thus emphasising the importance of the combined S1PR_{3/5} pathway (**Miron et al. 2010**). S1PR₃ parallels many activities of S1PR₁ and is implicated in Akt/mTOR and ERK1/2 signalling pathways (**Ogle et al. 2017**). The importance of both pathways in oligodendrocyte differentiation and remyelination has been extensively shown *in vivo* and *in vitro* (**Guardiola-Diaz et al. 2012, Ishii et al. 2013, Dai et al. 2014, Ishii et al. 2019**). A recent study by Ishii and colleagues (**2019**) shows both signalling pathways to work independently as well as cooperatively to regulate myelination processes. ERK1/2 pathway activation is also vital for oligodendrocyte survival, reducing oligodendrocyte apoptosis and promoting OPC differentiation (**Guardiola-Diaz et al. 2012, Ishii et al. 2013**). Furthermore, the Akt/mTOR pathway is a crucial element in directing PNS myelination onset and growth, and maintaining and myelin thickness in the CNS and PNS (**Figlia et al. 2017**). Together, such findings support the hypothesis that the beneficial effects observed with fingolimod treatment on myelination and lifespan, and conversely the lack thereof with siponimod, may be linked to activation of S1PR₃.

3.3. Siponimod regulates microglia in Twitcher mice in the absence of remyelination

Over the past 40 years, KD research has been governed by the “psychosine hypothesis”, which postulates that the accumulation of psychosine is the main culprit for the death of oligodendrocytes and the subsequent demyelination observed in KD (**Suzuki 1998**). Increasing evidence ascribes an important detrimental role in driving the pathology of KD to microglial activation (**Nicaise et al. 2016**). This novel “microglial hypothesis” is based on the presence of multinucleated macrophages termed globoid cells before overt signs of demyelination (**Potter et al. 2013, Nicaise et al. 2016**). While globoid cells are one of the hallmarks of KD pathology, their origins and function remains disputed. They are thought to originate either from CNS-resident microglia or from infiltrating peripheral macrophages. Most current therapies for KD are designed to prevent oligodendrocyte apoptosis, demyelination and neurodegeneration. However, the “microglial hypothesis” emphasises the importance of microglia activation and globoid cell infiltration as a primary focus of KD pathology. Microglia-mediated neuroinflammation is a common thread in propelling neuropathology. Research conducted using the GALC^{twi-5J} mouse have provided evidence that typical neuropathological hallmarks such as globoid cells, gliosis and psychosine accumulation precede severe demyelination and

axonal loss (**Potter et al. 2013**). Such findings support microglia and globoid cells as a therapeutic target to prevent early demise in KD. Microglia express all S1P receptor subtypes and their modulation has previously been shown to modulate microglial activation in models of traumatic brain injury and ischemia (**Zhang et al. 2008, Czech et al. 2009**). Here, we show siponimod to significantly reduce the number of Iba1 positive microglia in Twitcher mice. This result sits in line with a previous study reporting siponimod to reduce Iba1 positive microglia/macrophages in an EAE model (**Gentile et al. 2016**). Using an *in vitro* approach, that same study showed siponimod to reduce microglial activation by altering their cytokine release profile (**Gentile et al. 2016**). Similarly, administration of siponimod corresponded with a reduction in Iba1-positive microglia/macrophages following subpial cortical demyelination in EAE mice (**Ward et al. 2020**). Together, these data suggest that S1PR_{1/5} modulation may be sufficient to reduce microglia/macrophage activation.

3.4. Siponimod mediated immunomodulation and astrogliosis

KD research conducted over the past decades reveals a complex KD neuropathology involving the amalgamation of aberrant axonal, neuronal and myelination processes accompanied by exacerbating neuroinflammation (**Nicaise et al. 2016, Won et al. 2016**). Astrocytes have been implicated in the pathogenesis of KD, with elevated levels of astrocytic MMP3 expression at symptomatic onset in twitcher mice, which continues to rise with disease progression (**Ijichi et al. 2013, Claycomb et al. 2014**). Another study observed astrocyte levels to increase in twitcher mice, with elevated GFAP expression observed as early as 3 weeks of age (**Snook et al. 2014**). Such findings sit in line with our current results, where vimentin-labelled fibrous astrocytes in white matter tracts was significantly increased in twitcher mice. S1PR_{1/5} modulation using siponimod resulted in a significant decrease of those vimentin-positive fibrous astrocytes. Similarly, Gentile and colleagues (**2016**) reported siponimod to reduce astrogliosis in striatal slices in an EAE model. In our laboratory, we previously showed that the S1PR_{1/5} agonist, siponimod, was able to induce ERK and AKT phosphorylation in a concentration-dependent manner in both human and mouse astrocytes (**O'Sullivan et al. 2016**). This was also demonstrated by Osinde and colleagues (**2007**), demonstrating that S1PR₁ activation in particular mediates ERK phosphorylation in astrocytes. These data suggest that a lack of S1PR₃ activation did not impede the ability of siponimod to regulate ERK and AKT signalling pathways, which may rely on S1PR₁ and or S1PR₅.

We noted a discrepancy between vimentin and GFAP expression with siponimod treatment in twitcher mice. While vimentin positive fibrous astrocytes decreased in white matter tracts of

siponimod-treated twitcher mice, GFAP expression across groups remained the same. This could partially be explained by the fact that GFAP is expressed late in development of fibrous astrocytes, and may not constitute an adequate marker to investigate changes during early development (**Qian et al. 2000, Lundgaard et al. 2014**).

3.5. Siponimod – implications for future therapeutic strategies for KD

Our data show that siponimod treatment intermittently increased phenotypic abnormalities in twitcher mice and failed to significantly increase lifespan. This lack of effectiveness of siponimod may be explained by the lack of remyelination in twitcher mice. Additionally, consistent weight loss and decrease in size of visceral organs such as spleen and kidneys of twitcher mice, may contribute to ongoing and ultimately fatal disease progression in the peripheral system. Such observations suggest that correction of inflammatory component of KD by decreasing microglia and astrocyte activation is not sufficient to combat disease progression and phenotypic abnormalities. The “microglia hypothesis” implicates globoid cell infiltration and microglial activation as main contributing factors in the KD pathogenesis (**Nicaise et al. 2016**). While increasing evidence supports these inflammatory processes to play an exacerbating and disease driving function, our data seems to suggest that combating these factors within the CNS may not be sufficient to ameliorate disease progression. Psychosine circulates throughout the body and accumulates in all tissues, including the brain, spinal cord, kidney, liver, spleen and lung (**Svennerholm et al. 1980**). This multi-organ involvement suggests that correcting for CNS inflammation may result in modest and short-term symptomatic improvements. Long-term outcomes, however, would require a therapeutic strategy to target remyelinating processes in the CNS as well as regeneration of peripheral system using combination therapies (**Li and Sands 2014, Mikulka and Sands 2016**).

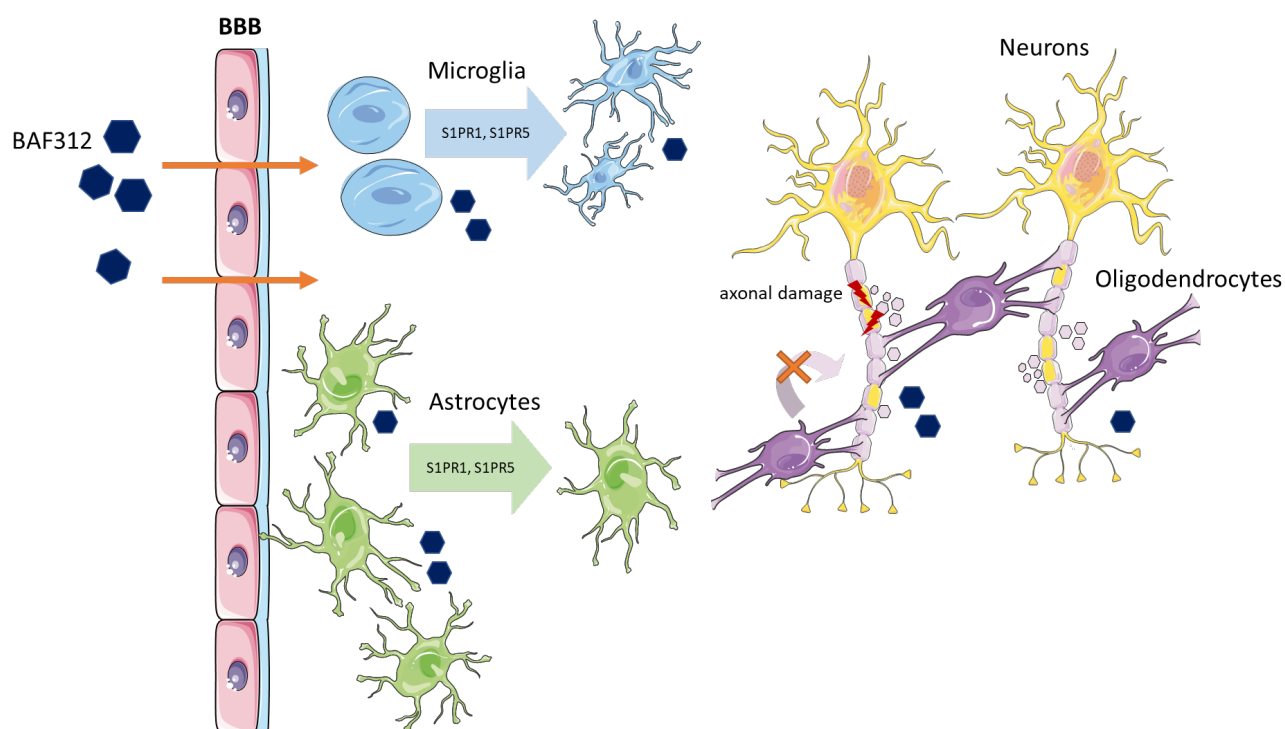


Figure 5.6 Summary figure of siponimod-mediated effects in twitcher mice

Fingolimod administered at PND21 was able to cross the BBB and act on CNS-resident cells. Selective S1PR_{1/5} modulation resulted in altered microglial morphology, as well as a reduction of vimentin-positive fibrous astrocytes in white matter tracts of twitcher mice. Interestingly, the absence of S1PR₃ agonism resulted in a lack of remyelination processes in twitcher mice. Similarly, no changes in myelin debris accumulation and axonal damage was observed with selective S1PR_{1/5} modulation.

Chapter 6 – Discussion

1. Study background

The past decade has witnessed an increasing interest in targeting the sphingosine 1-phosphate (S1P) axis as a therapeutic strategy, with numerous clinical trials and pre-clinical research in several neurodegenerative diseases (**O'Sullivan and Dev 2017**). The S1P axis refers to the signalling molecule S1P, its receptors as well as the proteins that synthesise, transport and degrade endogenous S1P. With the development of S1P receptor agonists and antagonists, receptor modulation has become a particularly promising therapeutic target. In addition to developing compounds that target S1P receptors, research efforts are aiming to modulate the S1P axis on other levels, for instance by inhibiting sphingosine kinases or S1P lyase activity, thus affecting S1P synthesis, degradation and export (**Kunkel et al. 2013**). The current study investigated two S1P receptor modulators, namely fingolimod (FTY720, Gilenya®) and siponimod (BAF312, Mayzent®). Fingolimod and siponimod are currently used for the treatment of relapsing-remitting and secondary progressive Multiple Sclerosis, respectively. Additionally, fingolimod is under investigation in clinical trials for a range of diseases, including acute stroke (NCT02002390), Rett syndrome (NCT02061137), amyotrophic lateral sclerosis (NCT01786174) and glioblastomas (NCT02490930). Previous studies from our lab have shown promising effects of both fingolimod and siponimod on myelination and inflammation in organotypic slice cultures and human astrocyte cultures (**O'Sullivan and Dev 2015, O'Sullivan et al. 2016, Misslin et al. 2017, O'Sullivan et al. 2017**). Together, such promising findings have prompted the present pre-clinical study, investigating the potential of fingolimod and siponimod in twitcher mice, a murine model of Krabbe's disease (KD). KD is a rare neurodegenerative disorder, predominantly affecting infants. It is a lysosomal storage disorder, caused by a defective gene *galc* encoding the hydrolase galactosylceramidase (GALC). The decreased enzymatic activity leads to a brain accumulation of the toxic metabolite psychosine, which promotes profound demyelination, severe oligodendrocyte death and neuroinflammation.

2. Summary of findings

Here we aimed to investigate the role of S1P receptor modulators as follows:

We first investigated the therapeutic benefit of fingolimod in twitcher mice. Animals were treated with a concentration of 1mg/kg/day starting at PND21 until they reached humane endpoints. Histologically, we found that fingolimod increased levels of MBP expression, without, however, affecting myelin debris accumulation. It also dampened neuroinflammatory response, with observed morphological changes of Iba1-labelled microglia/macrophages. Bergmann glia

in the molecular layer of the twitcher mouse cerebellum were significantly decreased as measured with the surrogate marker vimentin. This was increased with fingolimod treatment. White matter fibrous astrocytes labelled with vimentin were significantly increased, which was further enhanced with fingolimod treatment. Behaviourally, we found that fingolimod administration increased the lifespan of twitcher mice, when compared to untreated twitcher mice. Additionally, fingolimod had beneficial effects on weight, ataxic tremors, and hind limb paralysis in twitcher mice, when compared to untreated controls.

In a second study, we assessed the effects of fingolimod when administered neonatally. Based on our results from the previous chapter, we hypothesised that fingolimod could act prophylactically if administered before overt signs of disease progression and severe demyelination could occur in twitcher mice. We therefore treated twitcher mice and healthy wildtype animals with 1mg/kg/day of fingolimod, starting at PND5 until they reached humane endpoints. We found that prophylactic fingolimod administration did increase life span of twitcher mice, when compared to untreated animals. However, open field maze parameters measuring locomotor activity shows no significant improvements with fingolimod in twitcher mice compared to vehicle-treated controls. Indeed, wildtype animals treated with fingolimod performed significantly worse compared to vehicle-healthy wildtype littermates. Histologically, we observed no significant changes in myelin marker expression as measured with MBP and MOG. However, SMI32, a marker for non-phosphorylated neurofilament heavy, as well as degraded MBP were significantly decreased. This suggests that fingolimod had a positive effect on axonal damage, independent of remyelination processes. Fingolimod did not significantly modulate astrocyte reactivity as measured with vimentin and GFAP. However, fingolimod did significantly reduce Iba1-positive area, with morphological changes from amoeboid to ramified microglia in fingolimod treated twitcher mice.

Finally, we evaluated the effects of specific S1PR_{1/5} activation on myelination and inflammation using the S1PR₃ sparing compound siponimod. Based on previous findings from organotypic slice cultures, demonstrating that siponimod was able to reduce psychosine-induced demyelination (**O'Sullivan et al. 2016**), we wanted to investigate the therapeutic potential of siponimod *in vivo*. We therefore administered siponimod to twitcher mice, at a concentration of 1mg/kg/day starting at PND21. Interestingly, the more selective S1PR_{1/5} modulator siponimod was not able to replicate the promising findings observed with fingolimod treatment. Behaviourally, siponimod was not able to increase lifespan of twitcher mice, and treatment with siponimod did not result in significant improvements in weight, ataxia or hind limb paralysis. In line with our behavioural findings, siponimod did not significantly improve myelination levels

and myelin debris accumulation in the brain of twitcher mice when compared to healthy controls. However, we observed inflammatory improvements with reductions of Iba1-positive cells and vimentin immunofluorescence in white matter tracts of siponimod treated twitcher mice when compared to untreated twitcher animals. Nevertheless, this noted amelioration regarding the inflammatory state in the brain of twitcher mice did not significantly improve axonal damage and globoid cell infiltration.

3. The role of S1P receptors in myelination: implications for fingolimod and siponimod

Both human and rodent oligodendrocyte lineage cells respond to S1P receptor modulation through the expression of S1P receptor subtypes 1, 3 and 5 (**Miron et al. 2008**). S1PR₅ is highly expressed on mature oligodendrocytes, the activation of which has been linked to pro-survival effects in human and rodent mature oligodendrocytes (**Miron et al. 2008**). Remyelination via S1P receptors has mainly been attributed to S1PR₅ and/or S1PR₁ modulation (**Miron et al. 2010, Jackson et al. 2011, Sheridan and Dev 2012, O'Sullivan et al. 2016**). Previous data has shown both fingolimod and siponimod, but not AUY954 (S1PR₁ specific agonist), to increase MBP expression during the remyelination phase following LPC-induced demyelination in a rodent telencephalon (**Jackson et al. 2011**). Organotypic cerebellar slices treated with fingolimod and SEW2871 (S1PR₁ specific agonist) have also been shown to attenuate LPC-induced demyelination (**Sheridan and Dev 2012**). Similarly, in our laboratory, psychosine-induced demyelination in organotypic slices was attenuated through S1PR₁ and S1PR₅ modulation with siponimod (**O'Sullivan et al. 2016**). Here, we have shown promising effects on myelination with fingolimod treatment when administered at PND21 (**Béchet et al. 2020**). However, when administered earlier, starting at PND5, we found that the markers of myelin, MBP and MOG, did not increase in neonatal twitcher mice. This discrepancy may involve a differential developmental expression pattern of S1P receptor subtypes and the opposing role they play on immature and mature oligodendrocyte lineage cells during early development (**Jaillard et al. 2005, Jung et al. 2007, Miron et al. 2008**). Furthermore, fingolimod may act as a functional antagonist (**Oo et al. 2007, O'Sullivan et al. 2016**), which could sequester developmentally crucial receptors. S1P and its receptors are integral for proper neural and vascular development during embryonic stages (**Mizugishi et al. 2005**). Inhibition of endogenous S1P receptor signalling through abrogation of available receptors at early stages of development may thus inhibit beneficial effects of fingolimod during later development in twitcher mice.

Administration of the S1PR₃-sparing drug siponimod in this study did not result in significant improvements of myelin levels, as measured by MOG and MBP, in twitcher mice. The discrepancy between results yielded by both compounds, siponimod and fingolimod, suggests a crucial function for the S1PR₃ subtype. Indeed, a previous study has attributed fingolimod-mediated remyelinating processes to its activation of both S1PR₃ and S1PR₅ subtypes (**Miron et al. 2010**). S1PR₃ is implicated in Akt and MAP kinase signalling pathways, including ERK1/2 (**Pitson et al. 2003, Ogle et al. 2017**). Both of these pathways are involved in oligodendrocyte differentiation and survival, working independently as well as cooperatively to regulate myelination processes (**Guardiola-Diaz et al. 2012, Ishii et al. 2013, Ishii et al. 2019**). Together, such findings raise the hypothesis that the beneficial effects observed with fingolimod treatment at PND21 on myelination, and conversely the lack thereof with siponimod, may be in part linked to activation of S1PR₃ (**Figure 6.1**).

4. S1PR-mediated effects on microglial activation and polarisation

The main mechanism of action of both siponimod and fingolimod lies in their functional antagonism at S1P₁ receptors expressed by lymphocytes. Binding to those receptors leads to their internalisation and subsequent degradation (**Brinkmann et al. 2004, Pan et al. 2013**). Through this mechanism, both compounds are able to reduce immune cell infiltration into the CNS. Additionally, both compounds are able to cross the BBB and directly act upon S1PR expressing CNS resident cells, such as microglia. Microglia express S1P receptor subtypes 1, 2, 3 and 5 and thus constitute the perfect target for both siponimod and fingolimod (**Groves et al. 2013, O'Sullivan and Dev 2017**). As members of the innate immune system, microglia become activated upon exposure to damage- or pathogen-associated molecular patterns in the context of disease (**Lloyd and Miron 2019**). This activation involves morphological and gene transcriptional changes which has been associated with both damaging and regenerative responses (**Lloyd and Miron 2019**). Chronic pro-inflammatory microglial activation has neurotoxic capabilities, drives neuroinflammation and significantly contributes to the disease progression of many chronic neurodegenerative diseases and their associated models (**Nicaise et al. 2016, Gyengesi et al. 2019**). For instance, depletion of microglia has been shown to reduce pathology in experimental models of AD and EAE (**Spangenberg et al. 2016, Nissen et al. 2018**). On the other hand, microglia are also known to exhibit regenerative functions by supporting neurogenesis and contributing to remyelination processes (**Franklin and Ffrench-Constant 2017, Kanazawa et al. 2017**).

Based on these findings, suppressing neurotoxic effects of microglia or enhancing their neuroprotective effects would provide an effective therapeutic strategy to KD. Our findings reveal that both fingolimod and siponimod had a significant impact on microglial morphology and distribution. Microglia accumulated in their amoeboid shape surrounding demyelination areas in white matter tracts of twitcher mice, which was attenuated by both compounds. This finding sits in line with other observations, where fingolimod was able to modulate microglia reactivity (**Zhang et al. 2007, Jackson et al. 2011, Kim et al. 2011, Noda et al. 2013, Serdar et al. 2016**). Indeed, Jackson and colleagues (**2011**) showed fingolimod to reduce, but not abolish, ferritin levels, a marker of microglial activation. Such partial inhibition of microglial activation was correlated with enhanced remyelination following lysophosphatidyl choline-induced demyelination (**Jackson et al. 2011**). Similarly, Kim et al. (2011) show that the number of microglia accumulating in a cuprizone-induced model of demyelination was attenuated with fingolimod treatment. Interestingly, this study also showed that fingolimod did not elicit remyelination in the non-inflammatory cuprizone model, suggesting that neuroprotective and remyelinating effects are mediated by dampening the inflammatory response (**Kim et al. 2011**). In a neonatal model of hypoxia, fingolimod was found to inhibit microglial activation, inducing a ramified morphology of Iba1 positive cells and reducing pro-inflammatory cytokine panel (**Serdar et al. 2016**). Together, our findings and previous studies emphasise the efficacy of fingolimod- and siponimod-mediated S1PR modulation in reducing microglia reactivity, and their consequent potential in injury prevention and repair mechanisms following demyelinating insults.

5. The role of S1PRs in astrocytes and astrogliosis in Krabbe's disease

Altered astrocytic function is believed to be a major contributing factor to the progression of a number of neurological diseases, in particular, the pathogenesis of demyelinating disorders (**Molofsky et al. 2012**). Within the cerebellum, astrocytes are characterised by their ample functions and distinct morphological features (**Buffo and Rossi 2013**). In twitcher mice, astrocytes have been shown to undergo morphological changes associated with activation, and astrogliosis is evident in the hindbrain and cerebellum of twitcher mice as early as 3 weeks of age (**Snook et al. 2014**). These events coincided with microglia/macrophage recruitment, as well as a marked up-regulation of a large panel of cytokines/chemokines (**Snook et al. 2014**). Furthermore, astrocytic MMP3 was elevated in twitcher mice and continued to rise with disease progression (**Ijichi et al. 2013, Claycomb et al. 2014**). Such findings concur with results from our current study, where immunofluorescence in twitcher mice showed increased vimentin

expression in areas of demyelination. *In vitro*, both siponimod and fingolimod were shown to attenuate psychosine-mediated apoptosis and induce levels of pERK and pAKT in astrocytes (Osinde et al. 2007, Healy et al. 2013, O'Sullivan and Dev 2015). Here, we found fingolimod to enhance vimentin expression further in white matter tracts in twitcher mice. Kim et al. (2011) document similar findings with augmented astrogliosis when fingolimod was given to cuprizone-fed animals from week 4 to 9. In our hands, siponimod attenuated the vimentin immunofluorescence in white matter tracts. This discrepancy between fingolimod and siponimod on astrogliosis in twitcher mice could be explained by their differing affinity and/or mechanism of action on S1PR₁ and S1PR₃, both of which are expressed on astrocytes (Mullershausen et al. 2007, Dusaban et al. 2017). S1PR₃ upregulation in astrocytes has been linked to ongoing inflammatory responses (Wu et al. 2008, Dusaban et al. 2017). Deletion of S1PR₃, along with sphingosine kinase 1 (SphK1), resulted in decreased astrocyte proliferation and gliosis in a murine model of Sandhoff disease (Wu et al. 2008). Similarly, SphK1 and S1PR₃ axis were shown to be upregulated in LPS activated astrocytes (Fischer et al. 2011). Such results suggest that S1PR₃ mediated signalling with fingolimod in astrocytes could play a role in the establishment and maintenance of astrocyte activation. Several studies have described beneficial effects with functional antagonism of astrocytic S1PR₁ (Mullershausen et al. 2009, Choi et al. 2011, Healy et al. 2013, O'Sullivan et al. 2016, Chen et al. 2019). For instance, deletion of S1PR₁ on astrocytes correlates with improved clinical outcomes, reduced demyelination, axonal loss and astrogliosis in an EAE model (Choi et al. 2011). Both siponimod and fingolimod induce internalisation of astrocytic S1PR₁ (Mullershausen et al. 2009, O'Sullivan et al. 2016), which could account for the reduced astrogliosis observed with the S1PR₃ sparing compound siponimod. Increased astrogliosis with fingolimod on the other hand may be linked to its agonistic activity on S1PR₃, resulting in prolonged inflammation.

6. S1P mediated clearance of myelin debris in twitcher mice

The ability to clear myelin debris is a critical component of remyelination, a phenomenon known to be impaired in demyelinating diseases such as multiple sclerosis (Lampron et al. 2015, Lloyd and Miron 2019). We therefore examined the extent of myelin debris using a marker against a degraded epitope of MBP (dMBP). As expected, we observed a significant increase in myelin debris surrounding white matter tracts. Neither fingolimod nor siponimod were able to significantly reduce myelin debris when administered post-weaning (PND21). Interestingly, fingolimod did significantly reduce myelin debris accumulation when administered at PND5, which was paralleled by decreased axonal damage. This suggests that prophylactic

administration of fingolimod is more effective in clearing myelin debris, or preventing severe myelin debris accumulation.

S1P and its receptors have been implicated in phagocytosis of both pathogens and cellular debris (**Gude et al. 2008, McQuiston et al. 2011**). S1P is produced by cells upon induction of apoptosis and attracts phagocytes to clear remaining cellular debris (**Gude et al. 2008, Weigert et al. 2019**). Additionally, S1P has been shown to prime macrophages and promote phagocytosis of cell debris (efferocytosis) (**Luo et al. 2016**). Interestingly, inhibition of sphingosine kinases in alveolar macrophages has been shown to reduce efferocytosis in patients suffering from chronic obstructive pulmonary disease (**Tran et al. 2016**). Fingolimod treatment reversed this inhibited efferocytosis by promoting sphingosine kinase functionality (**Tran et al. 2016**). S1PR-mediated signalling may also play a role in phagocytosis, and has predominantly been linked to S1PR₂ (**McQuiston et al. 2011**). The lack of myelin debris clearance observed with siponimod and fingolimod at PND21 could be due to the failure of both compounds to induce S1PR₂ signalling. Prophylactic administration of fingolimod in twitcher mice, however, did reveal reduced myelin debris accumulation. Early fingolimod-mediated action could accelerate the microglial shift in phenotype toward M2 polarisation, which has been associated with improved phagocytosis (**Park et al. 2016, Qin et al. 2017**). During stages of remyelination, microglial cells go through several critical phenotypic stages (**Lloyd and Miron 2019**). While early phases of repair and OPC recruitment are characterised by an initial (M1) pro-inflammatory phenotype (iNOS⁺, TNF⁺ and CD16-CD32⁺), subsequent phases of oligodendrocyte differentiation and remyelination are dominated by a regenerative (M2) microglial phenotype (ARG1⁺, IGF1⁺ and CD206⁺) (**Miron et al. 2013**). This phenotypic transition is imperative in aiding remyelination, as persistent pro-inflammatory microglial activation with an iNOS⁺ and CD16-CD32⁺ dominant phenotype as well as depleted presence of CD206⁺ population is associated with impaired remyelination (**Miron et al. 2013**). Previous studies have shown fingolimod to enable this phenotypic shift from M1 to M2 polarisation, with reduction in pro-inflammatory cytokines (**Jackson et al. 2011, Qin et al. 2017**).

7. Treatment for Krabbe's Disease – finding the combination

Although KD is a monogenic disease, its pathology is characterised by a complex biochemical, histological and clinical phenotype. The galactosylceramidase deficiency observed in KD results in a widespread central and peripheral nervous system degeneration, with severe repercussions on cognitive and motor development (**Wenger et al. 2016**). The extensive range and rapid progression of symptoms are indicative of the multimodal implications of KD, with widespread

axonopathy and hypomyelination throughout the peripheral nervous system of GALC^{twi-5J} homozygotes, including the enteric and autonomic systems dysregulation (**Potter et al. 2013**). Similarly, we observed wide-ranging deterioration in our twitcher mice, involving both central and peripheral systems. Severe demyelination, inflammation and globoid cell infiltration in the CNS were accompanied by peripheral nerve demyelination and inflammation within sciatic nerves. We also showed fingolimod and siponimod to improve neuropathological abnormalities in the CNS of twitcher mice, with reduced microglia activation and astrogliosis, reduced axonal damage and increased myelination. However, these ameliorations were not mirrored in the peripheral system nor in our behavioural outcome measures. While we observed modest increases in lifespan with fingolimod and intermittent improvements in motor function with fingolimod and siponimod, weight measurements of twitcher mice ultimately converged back to that of untreated littermates, and animals eventually developed ataxia, flaccid paralysis of hindlimbs likely resulting from the continuing peripheral nerve degeneration and demyelination.

Failure to correct the PNS remains a considerable challenge for long-term clinical improvements in both animal models and humans (**Escobar et al. 2005, Wenger et al. 2016**). Thus, our observations, as well as previous studies, emphasise the need to target both central and peripheral systems to provide a more effective therapeutic strategy (**Galbiati et al. 2007, Marshall et al. 2018**). To date, single therapies for murine KD have investigated a wide range of experimental therapeutic avenues, including substrate reduction therapy (**LeVine et al. 2000**), enzyme replacement therapy (**Lee et al. 2007**), and gene therapy (**Lin et al. 2005, Rafi et al. 2012**). While some of these therapies significantly enhanced lifespan of animals, no treatment to date has resulted in a complete correction of clinical pathological features (**Li and Sands 2014**). This lack of success emphasizes the need for combination therapies targeting multiple aspects of the disorder in the CNS as well as PNS. An increasing number of studies show the importance of multi-modality therapies within the context of KD (**Li and Sands 2014, Mikulka and Sands 2016**). For instance, a combination therapy using bone marrow transplant and AAV2/5-GALC-mediated gene therapy was able to extend significantly twitcher lifespan, compared to AAV2/5-GALC alone (**Lin et al. 2005**). Similarly, Marshall and colleagues (**2018**) demonstrated the increased efficacy of adeno-associated virus (AAV)9-based gene therapy in tandem with bone marrow transplant. While bone marrow transplant alone was unable to correct neuroinflammation within the CNS or macrophage infiltration within the sciatic nerves, global AAV9-GALC injections (intracranial, intrathecal and intravenous injections) and AAV9-GALC combined with bone marrow transplant resulted in significant long-term improvements

to behavioural deficits, pathophysiological abnormalities and survival (**Marshall et al. 2018**). Such promising findings from multi-modality approaches highlight the need for further research into the combination of experimental therapies to create the optimal synergistic interaction for the treatment of KD.

8. Limitations and Future Directions

The current study examined the protective effects of S1P receptor modulation in twitcher mice, a murine model of the devastating leukodystrophy Krabbe's Disease. We highlighted several beneficial effects on myelination and inflammation with S1PR modulation in the CNS. However, some of the limitations observed over the course of our studies are worthy of mention. Firstly, psychosine accumulation and defective GALC activity are known to be the main cause of disease progression (**Graziano and Cardile 2015**). However, GALC activity or psychosine-levels in tissue of twitcher mice were not measured in our study. We hypothesise that the lack of effectiveness of S1PR modulation in the periphery may be related to increased psychosine levels throughout the body (**Svennerholm et al. 1980**). This multi-organ involvement likely contributes to the ultimately fatal disease progression in the peripheral system of twitcher mice. Further analysis measuring psychosine accumulation by Liquid Chromatography–tandem Mass Spectrometry would be needed to support this hypothesis.

In this thesis, we have used fluorescent intensity of myelin markers such as MBP and MOG as a surrogate indicator of myelin state. However, these proteins are also present in non-myelination oligodendrocytes and cell debris and may consequently provide an incorrect estimation of functional myelin. Additionally, this technique does not address the thickness or compaction of the myelin sheath. Considering this, the use of electron microscopy to corroborate our findings would provide more in-depth information regarding the state of the myelin sheath. Nerve conduction velocity studies could also be carried out to test the peripheral nerve integrity and functional quality in twitcher mice, as previously described (**Walsh et al. 2015**).

A behavioural limitation of our study involved the use of observational scoring sheet. While attempts were made to limit observer bias by blinding the researcher to the genotype of animals, the latter became obvious due to the overt phenotype of twitcher mice. In order to limit bias, behavioural assessments should include objective measures of locomotion. Studies investigating various therapeutic strategies in twitcher mice have described objective measurements of motor function (**Ripoll et al. 2011, Scruggs et al. 2013**). The wire hang

manoeuvre allows assessment of hindlimb strength and the rotarod test measures balance, coordination and strength. Additionally, hind stride length can be measured by applying food colouring to the paws of the animals and allowing them to walk through a tube lined with graph paper. Such tests would provide valuable information, in addition to our Open Field Maze, regarding subtle differences in locomotion of twitcher mice.

S1P receptor modulators have revealed promising in several neurodegenerative diseases (**O'Sullivan and Dev 2017**). Our current findings indicate that S1P receptors could be a potential target in the treatment of KD, with reported improvements on myelination and inflammation in the CNS. However, failure to correct the PNS resulted in modest correction of clinical pathological features, with a limited increase in lifespan. We hypothesise that S1P receptor modulators used in conjunction with therapies known to improve PNS, such as AAVrh10-GALC gene therapy, would result in more promising results with regards to clinical outcome measures. Gene therapy was able to deliver normal levels of GALC activity within the CNS and PNS, and significantly reduced behavioural phenotypes and lifespan in twitcher mice (**Rafi et al. 2012, Rafi et al. 2015**). The synergistic interaction between AAVrh10-GALC and S1P receptor modulation could thus highly benefit twitcher mice and provide long-lasting effects on phenotypic abnormalities and lifespan.

9. Closing remarks

Demyelinating disorders such as KD have devastating effects on the lives of the patient and their family. Although the disease is rare, clinical practice is in urgent need for a more efficacious and non-invasive treatment that will improve the quality of life of affected infants. The neuroprotective spectrum of S1P receptor modulators has been illustrated within the context of a variety of neurodegenerative diseases (**O'Sullivan and Dev 2017**). Fingolimod and siponimod, two S1P receptor agonists, have been approved for treatment of relapsing-remitting and secondary-progressive MS, respectively (**Kappos et al. 2015, Kappos et al. 2018**). Fingolimod is also in clinical trials for the treatment of Rett syndrome (**Naegelin et al. 2015**). Additionally, the efficacy of fingolimod has been described in a number of animal models, including models of Alzheimer's disease (**Aytan et al. 2016**), spinal cord injury (**Norimatsu et al. 2012**), ischaemic stroke (**Kraft et al. 2013**) and Huntington's disease (**Di Pardo et al. 2014**). Here, we showed both fingolimod and siponimod to modestly improve biochemical abnormalities in twitcher mice, a murine model for KD. While we had hoped for more promising effects on lifespan and phenotypic abnormalities, our results emphasise the importance of multi-organ

degeneration in KD. Although fingolimod and siponimod were able to provide partial efficacy within the CNS, their failure to correct PNS and visceral organ damage underlines their relevance in the pathology of KD. Data presented in the current study warrant further investigation into combination therapies of KD that would target both CNS as well as the peripheral systems. Furthermore, administration of fingolimod from PND5 onwards in our study raised some concerns regarding potential side-effects during early neonatal development. These preliminary findings require further investigation into the role of S1P and its receptors during embryonic and neonatal development. In closing, we consider KD as a multimodal illness that will require targeting of multiple systems and perhaps a set of drugs that may be used in a combinatorial manner to target the various mechanisms of this illness.

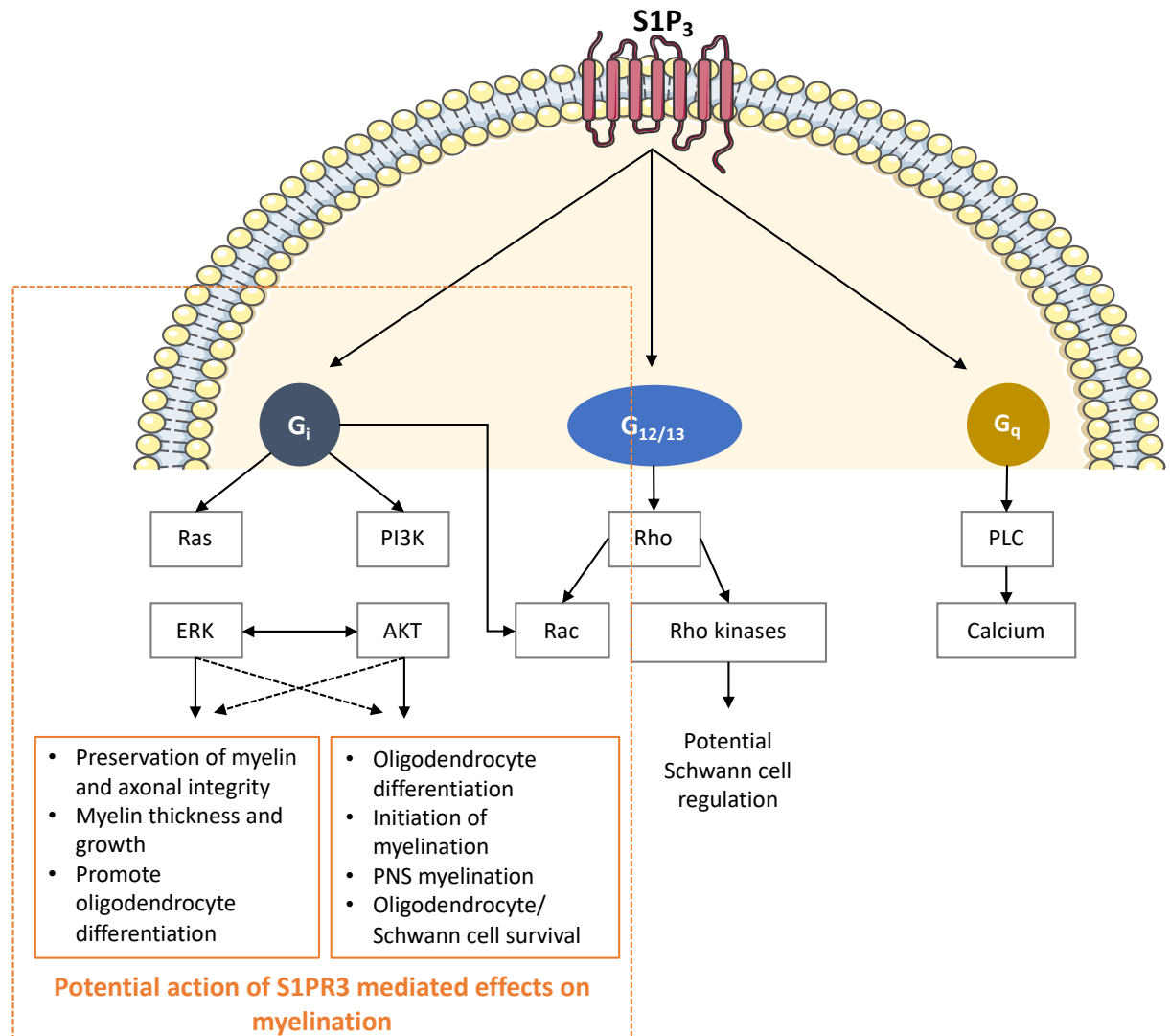


Figure 6.1 Potential role of S1PR₃ on myelination

Oligodendrocyte lineage cells respond to S1PR modulation through receptor subtypes 1, 3 and 5 (Miron et al. 2008). S1PR₃ activate G_i proteins that, in turn, enable downstream signalling ERK1/2 and Akt pathways. S1PR₃ modulation through the ERK signalling pathways could subsequently result in preservation of myelin and axonal integrity, increased myelin thickness and growth and oligodendrocyte differentiation. Additionally, S1PR₃-mediated downstream signalling via Akt is related to oligodendrocyte differentiation, myelination initiation, PNS myelination and survival of myelinating cells in CNS and PNS.

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Appendix I

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Appendix II

	Genotype	Treatment
PND25	Total distance travelled (m)	F(1,31)=7.410, *p=0.0106
	Mean velocity (m/s)	F(1,31)=7.454, *p=0.0103
	Maximum velocity (m/s)	F(1,31)=6.249, *p=0.0179
	Time mobile (%)	F(1,31)=3.845, p=0.0586
	Centre entries	F(1,31)=11.44, **p=0.0020
	Corner time (%)	F(1,31)=21.20, ****p<0.0001
PND28	Total distance travelled (m)	F(1,36)=24.3, ****p<0.0001
	Mean velocity (m/s)	F(1,36)=24.82, ****p<0.0001
	Maximum velocity (m/s)	F(1,36)=41.94, ****p<0.0001
	Time mobile (%)	F(1,36)=19.62, ****p<0.0001
	Centre entries	Non parametric test
	Corner time (%)	F(1,36)=23.83, ****p<0.0001
PND30	Total distance travelled (m)	F(1,30)=25.25, ****p<0.0001
	Mean velocity (m/s)	F(1,30)=25.14, ****p<0.0001
	Maximum velocity (m/s)	F(1,30)=29.67, ****p<0.0001
	Time mobile (%)	Non parametric test
	Centre entries	Non parametric test
	Corner time (%)	F(1,30)=16.11, ***p=0.0004

Supplementary Table 1. Genotype- and treatment-related differences in computer-automated spatial and motor parameters observed between PND25-30.

	WT^{Veh}	WT^{FTY}	Tw^{Veh}	Tw^{FTY}						
Spatial Parameters	Mean ±SEM	Mean ±SEM	Mean ±SEM	Mean ±SEM	WT^{Veh} vs WT^{FTY}	WT^{Veh} vs Tw^{Veh}	WT^{Veh} vs Tw^{FTY}	WT^{FTY} vs Tw^{Veh}	WT^{FTY} vs Tw^{FTY}	Tw^{Veh} vs Tw^{FTY}
Number of animals	8	8	11	8						
Total distance travelled (m)	12.32 ±1.61	11.89 ±1.28	10.27 ±1.33	6.192 ±1.38	ns	ns	*p=0.0297	ns	*p=0.0481	ns
Mean velocity (cm/s)	4.1 ±0.5	4.0 ±0.4	3.4 ±0.4	2.1 ±0.5	ns	ns	*p=0.0292	ns	*p=0.0444	ns
Maximum velocity (cm/s) ¹	29.5 ±3.2	29.5 ±2.4	26.2 ±2.8	19.8 ±0.8	ns	ns	*p=0.0290	ns	**p=0.0052	ns
Time mobile (%)	72.99 ±5.06	68.4 ±4.33	68.99 ±5.73	49.65 ±7.12	ns	ns	*0.0462	ns	ns	ns
Centre entries	15.38 ±1.59	8.63 ±2.22	8.09 ±1.67	4.13 ±1.23	ns	*p=0.0217	***p=0.0006	ns	ns	ns
Corner time (%)	24.33 ±2.36	35.48 ±2.64	45.91 ±5.43	58.13 ±6.26	ns	*p=0.0125	***p=0.0002	ns	*p=0.0151	ns

Supplementary Table 2. Characteristics of treatment groups across each parameter measured at postnatal day 25

¹ Non-parametric data analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test

	WT ^{Veh}	WT ^{FTY}	Tw ^{Veh}	Tw ^{FTY}						
Spatial Parameters	Mean ±SEM	Mean ±SEM	Mean ±SEM	Mean ±SEM	WT ^{Veh} vs WT ^{FTY}	WT ^{Veh} vs Tw ^{Veh}	WT ^{Veh} vs Tw ^{FTY}	WT ^{FTY} vs Tw ^{Veh}	WT ^{FTY} vs Tw ^{FTY}	Tw ^{Veh} vs Tw ^{FTY}
Number of animals	10	9	10	11						
Total distance travelled (m)	16.63 ±2.5	9.43 ±1.08	5.74 ±1.58	4.07 ±0.96	*p=0.0231	***p=0.0002	****p<0.0001	ns	ns	ns
Mean velocity (cm/s)	5.5 ±0.8	3.2 ±0.4	1.9 ±0.5	1.4 ±0.3	*p=0.0289	***p=0.0002	****p<0.0001	ns	ns	ns
Maximum velocity (cm/s)	38.4 ±2.7	28.6±2.4	17.5 ±3.2	16.7 ±1.5	ns	****p<0.0001	****p<0.0001	*p=0.0226	*0.0104	ns
Time mobile (%)	77.92 ±6.26	59.84 ±5.38	43.46 ±8.72	32.09 ±6.89	ns	**p=0.007	***p=0.0002	ns	*p=0.0402	ns
Centre entries ²	11.6 ±1.38	9.33 ±1.38	6.0 ±2.4	2.64 ±1.03	ns	ns	ns	ns	*p=0.0195	ns
Corner time (%)	38.13 ±2.6	32.86 ±4.09	54.10 ±8.21	72.64 ±5.76	ns	ns	***p=0.0005	ns	p=0.0001	ns

Supplementary Table 3. Characteristics of treatment groups across each parameter measured at postnatal day 28

² Non-parametric data analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test

	WT^{Veh}	WT^{FTY}	Twⁱ^{Veh}	Twⁱ^{FTY}						
Spatial Parameters	Mean ±SEM	Mean ±SEM	Mean ±SEM	Mean ±SEM	WT^{Veh} vs WT^{FTY}	WT^{Veh} vs Twⁱ^{Veh}	WT^{Veh} vs Twⁱ^{FTY}	WT^{FTY} vs Twⁱ^{Veh}	WT^{FTY} vs Twⁱ^{FTY}	Twⁱ^{Veh} vs Twⁱ^{FTY}
Number of animals	8	9	6	11						
Total distance travelled (m)	13.45 ±2.27	7.42 ±0.89	4.54 ±0.95	2.79 ±0.79	*p=0.0148	***p=0.0009	****p<0.0001	ns	ns	ns
Mean velocity (cm/s)	4.5 ±0.8	2.5 ±0.3	1.5 ±0.3	0.9 ±0.3	*p=0.0167	***p=0.0010	****p<0.0001	ns	ns	ns
Maximum velocity (cm/s)	32.7 ±3.6	30 ±2.4	17.3 ±1.6	16 ±2.2	ns	**p=0.0044	***p=0.0003	*p=0.0190	**p=0.0018	ns
Time mobile (%) ³	64.13 ±6.4	51.32 ±4.21	42.7 ±5.96	28.54 ±6.79	ns	ns	**p=0.0099	ns	ns	ns
Centre entries ³	12.63 ±2.85	9.33 ±1.41	3.33 ±1.63	1.64 ±1.16	ns	ns	**p=0.0019	ns	**p=0.0043	ns
Corner time (%)	64.13 ±6.4	36.11 ±3.56	68.91 ±6.37	81.56 ±6.81	*p=0.0148	ns	ns	**0.0077	****p<0.0001	ns

Supplementary Table 4. Characteristics of treatment groups across each parameter measured at postnatal day 30

³ Non-parametric data analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test