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The modulation of S1P receptor signalling and demyelination

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

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(2014)

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List of Abbreviations

Sphingosine 1-phosphate (S1P) - Sphingosine 1-phosphate receptor (S1PR) - Central nervous system (CNS) – Multiple sclerosis (MS) - G-protein coupled receptor (GPCR) - Experimental autoimmune encephalomyelitis (EAE) - Blood brain barrier (BBB) - Phosphorylated FTY720 (pFTY720) - Sphingosine Kinase (SphK) - High density lipoprotein (HDL) - Extracellular-signal-regulated kinase (ERK) - Vascular endothelial growth factor (VEGF) - Natural killer cell (NK cell) - Adenylate Cyclase (AC) - Phospholipase C (PLC) - Cyclic adenosine monophosphate (cAMP) - Phosphoinositide 3-kinase (Pi3K) - Protein Kinase C (PKC) - Ras homolog A (RhoA) - cAMP response element-binding protein (CREB) - Lymph node (LN) - Regulatory T cell (T reg) - Interleukin (IL) - Interferon (IFN) - Non-obese diabetic (NOD) - Transforming growth factor-β (TGF-β) - Knockouts (KO) - Human umbilical vein endothelial cells (HUVEC) - Leukaemia associated RhoGEF (LARG) - Vascular cell adhesion molecule 1 (VCAM-1) - Intercellular adhesion molecule 1 (ICAM-1) - Neural progenitor cells (NPCs) - Pertussis toxin (PTx) - Pheochromocytoma (PC12) - Nerve growth factor (NGF) - Arachidonic acid (AA) - Basic fibroblast growth factor (bFGF) - Lipopolysaccharides (LPS) - Platelet derived growth factor (PDGF) - Oligodendrocyte precursor cell (OPC) - Mitogen activated protein kinase (MAPK) - Platelet-derived growth factor (PDGF) - Neurotrophin-3 (NT-3) - Receptor tyrosine kinases (RTK) - Small interfering RNA (siRNA) - Residual pluripotent stem (rPS) - Cerebrospinal fluid (CSF) - Lysophosphatidylcholine (LPC) - Glial fibrillary acidic protein (GFAP) - Myelin basic protein (MBP) - Tumour necrosis factor alpha (TNF-α) - Dark Agouti (DA) - Endothelial cell adhesion molecules (ECAMs) - Myelin oligodendrocyte glycoprotein (MOG) - Relapsing Remitting (RRMS) - Secondary Progressive (SPMS) - Primary Progressive (PPMS) - Primary Relapsing (PRMS) - Ras-binding domain (RBD) - Cysteine-rich domain (CRD) - MAPK/ERK kinase (MEK) - Kinase suppressor of Ras-1 (KSR1) - Connector–enhancer of KSR (CNK) - MEK partner 1 (MP1) - Ras-kinase inhibiting protein (RKIP) - MAPK organizer-1 (MORG-1) - Son of sevenless (SOS) - Ethyleneglycoltetraacetic acid (EGTA) - Ethylenediaminetetraacetic acid (EDTA) - Mammalian target of rapamycin (mTOR) - Phosphatidylinositol 4,5-bisphosphate (PIP_2) - Phosphatase and tensin homolog (PTEN) - Phosphoinositide-dependent kinase-1 (PDK1) - mTOR complex 1 (mTORc1) - Regulatory-associated protein of mTOR (Raptor) - Mammalian lethal with SEC13 protein 8 (mLST8, also known as GβL) - Proline-rich AKT1 substrate 1 (PRAS40) - DEP domain-containing mTOR-interacting protein (DEPTOR) - Rapamycin-insensitive companion of mTOR (RICTOR) - Stress-activated MAP-interacting protein (SIN1) -

Protein observed with RICTOR (PROTOR) - Insulin-like growth factor 1 (IGF-IR) - Adenosine tri-phosphate (ATP) - Uridine 5'-triphosphate (UTP) - Human epidermal growth factor receptor 2 (HER-2) - Major histocompatibility complex class I (MHC I) - Endothelial Differentiation Gene-1 (EDG-1) - Lysophosphatidic acid (LPA) - Grb2-associated binding protein (GAB) - Antigen presenting cells (APCs).

Scientific Abstract

Sphingosine 1-phosphate receptors are a family of G-protein coupled receptors (GPCRs) that consists of five subtypes (S1PR1-5). These receptors have emerged as therapeutic targets in multiple sclerosis (MS) due to the efficacy of FTY720, also called fingolimod (Gilenya™, Novartis). FTY720 is phosphorylated to its active form (pFTY720) by sphingosine kinases (SphKs) and is an agonist for all S1PRs with the exception of S1PR2. pFTY720 is an immunomodulator that inhibits lymphocyte entry into the periphery and consequent passage into the CNS, thus reducing inflammation and demyelination. Under normal circumstances, lymphocytes exit the lymph node based on the positive concentration gradient of S1P that exists between the interstitial space of the lymph node and the efferent lymph. pFTY720 has been shown to prevent lymphocyte egress from lymph nodes by activating S1PR1s and causing their persistent internalisation, thus making the lymphocyte insensitive to the S1P gradient. Furthermore, FTY720 crosses the blood-brain barrier (BBB) and is phosphorylated by SphKs therein. For this reason, studies have been conducted examining the effects of S1PR modulation on neurons and glia. These studies suggest that effects of pFTY720 within the CNS may contribute to the benefits of the compound in MS treatment. This current thesis examined the effects of S1PR modulation on astrocytes as well as immune cells that have passed into the brain. The data demonstrates that S1PR1-activation induces the phosphorylation of ERK and Akt in astrocytes. A peptide designed to mimic the C-terminal of S1PR1, MNP301, was shown to inhibit pERK and also regulate the crosstalk between pERK and pAkt. Regulation of pERK/pAkt crosstalk by MNP301, as well as the MEK inhibitor U0126, resulted in enhanced Akt phosphorylation upon S1PR1 activation as well as increased astrocyte migration. In addition, pFTY720 reduced the ability of MOG-reactive splenocytes to induce demyelination in a cerebellar slice culture while also reducing IFN-γ and IL-6 released by these splenocytes. Overall, these data support the hypothesis that S1PR modulation may exert beneficial effects directly within the CNS, not only through actions on glia but also by regulating the function of peripheral immune cells that have crossed the BBB. Moreover, this thesis provides further knowledge on the mechanism of action of pFTY720 and supports the use of S1PRs as drug targets in other neuroinflammatory illnesses.

Lay Abstract

Multiple sclerosis (MS) is a disease that affects the cells that form the insulating layer that covers cells called neurons in the brain and spinal cord (central nervous system, CNS). Neurons are the primary cell type that send and receive signals to, from and within the CNS. This insulating layer is called myelin and removal of this insulation is called demyelination. In MS, demyelination is thought to occur when immune cells, that ordinarily stay out of the brain but protect the rest of the body from outside attack , pass into the CNS and attack the myelin layer. These attacks damage myelin and impair the signals sent by neurons. Symptoms seen in MS include numbness, muscle weakness, loss of vision and paralysis. A new treatment for MS called FTY720, or fingolimod (Gilenya™, Novartis) prevents immune cells from passing into the brain and reducing the severity and frequency of attacks. FTY720 has also been shown to act on cells within the CNS and it is thought that these effects may also be of benefit to patients. This current study examines effects of FTY720 on a type of brain cell known as astrocytes as well as effects of the compound on immune cells that have already passed into the brain. We find that FTY720 works by stopping harmful immune cells in the brain and also by directly altering astrocyte cell function which may reduce demyelination.

Aims and Hypothesis

This study aimed to address two main questions:

Firstly, it asked, how can the pathway selective modulator, MNP301, regulate S1PR signalling and what are the functional outcomes of this modulation? The hypothesis was that MNP301, will regulate S1PR signalling (such as pERK and pAkt) and this in turn will alter astrocyte cell function (such as astrocyte migration) These studies were the basis of Chapter 3 and 4.

Secondly, this study aimed to explore the role of S1PR modulation on immune-cell mediated demyelination (chapter 5). It was hypothesised that MOG-reactive immune-cells would induce demyelination of cerebellar organotypic slices in a co-culture system and that pFTY720 would inhibit this demyelination. This was the focus of Chapter 5.

Value of Research

There is much debate in the field of sphingosine 1-phosphate biology as to whether the benefits of treatment with the novel oral compound FTY720 in MS are solely due to peripheral immunomodulatory effects or whether direct effects of the compound on glia within the CNS may also contribute. This current study examined novel ways of regulating glial function via S1PR1 modulation. By making use of a selective pathway-inhibitor, MNP301, it was possible to differentially regulate signalling pathways downstream of S1PR1 activation. This peptide provides a level of specificity of inhibition that is not available with classical agonists and antagonists. This thesis, shows for the first time, that pathway specific modulation of S1PRs is important for astrocyte migration. Moreover, the findings of this thesis support the idea that MNP301 may prove to be a valuable research tool and should be explored for its use *in vivo*.

This study also examined how S1PR-modulation on peripheral immune cells, already within the CNS, may have added benefit in MS therapy. The data showed that pFTY720 inhibits demyelination induced by MOG-reactive immune cells resident in a brain slice. These data give an insight as to a potential unexplored benefit of S1PR modulation in MS, whereby pFTY720 may reduce inflammation and demyelination induced by peripheral immune cells that pass into the CNS at disease onset but prior to initiation of treatment.

Outputs

Papers

Pritchard, A.J. & Dev, K.K., 2013. The role of S1P receptors in the treatment of demyelinating diseases. *Future Neurology*, 8(5), pp.569–581.

Healy, Luke M, Sheridan, Graham K, Pritchard, Adam J et al. 2013. Pathway specific modulation of S1P1 receptor signalling in rat and human astrocytes. *British journal of pharmacology*, 169(5), pp.1114–1129

Pritchard A.J, Mir, A.K & Dev K.K. S1P receptor modulation attenuates splenocyte-induced demyelination in cerebellar slice cultures. (Submitted)

Pritchard A.J, Lockhart, A & Dev K.K. Pathway specific regulation of S1PR signalling and astrocyte migration (in preparation)

Presented Talks

Pritchard, A.J. 2013 Mechanisms of demyelination in a brain slice, 3rd Annual Frontiers in Neurology Meeting.

Presented Posters

The effects of S1P receptor modulation in a model of T cell induced demyelination, 2012 FENS Forum, Barcelona.

The effect of S1P receptor modulation in a T cell induced demyelination model, 2012 2nd Annual Block MS Meeting, Dublin

The Role of S1P Receptors in a T cell–induced Demyelination Model. 2011 IBRO 8th World Congress, Florence.

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Neuroscience Ireland student travel award 2012 (€250)

Trinity College Dublin student travel award 2012 (€250)

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Chapter 1 Introduction

1 Introduction to sphingosine 1-phosphate receptors

Sphingosine 1-phosphate (S1P) receptors (S1PRs) have been described over the last decade as important modulators of immune cell migration, particularly T cells (Matloubian et al. 2004; Zhi et al. 2011; Pappu et al. 2007). Recently, a novel S1PR agonist known as Fingolimod or FTY720 (Gilenya™, Novartis), has shown excellent efficacy in the treatment of relapsing remitting multiple sclerosis (MS) (Kappos et al. 2010). The phosphorylated form of FTY720 (pFTY720) modulates the S1P receptor subtype 1 (S1PR1) to cause sequestration of T cells within the lymph nodes (Matloubian et al. 2004) and therefore prevents T cell entry into the peripheral circulation and consequently the central nervous system (CNS). In addition to the expression of S1PRs in immune cells, this receptor family is also expressed in many other cell types including those of the cardiovascular system (Takuwa et al. 2009) and the CNS **(Table 1.1)** (Dev et al. 2008).

The potential for S1PRs as drug targets in the CNS has been highlighted recently by a number of studies. It has been shown that all brain cells in the CNS express S1PRs at varying degrees (Dev et al. 2008; Pritchard & Dev 2013) **(Table 1.1)**. Moreover, lipophilic FTY720 crosses the blood brain barrier (BBB), enters the CNS, where it is phosphorylated to its active form, and is found in white matter when given orally (Foster et al. 2007). In the CNS, both endogenous S1P and pFTY720 have been shown to induce functional changes in astrocytes, neurons, microglia and also oligodendrocytes (Ling et al. 2010; Pritchard & Dev 2013). It has been shown that genetic ablation of S1PR1 in astrocytes is somewhat protective in experimental allergic encephalomyelitis (EAE) (Choi et al. 2011). In addition, a selective S1PR1 agonist (CYM5442), which persists in the CNS but does not cause sustained lymphopenia has also been shown to attenuate EAE (Gonzalez-Cabrera et al. 2012). Collectively, these data have lead researchers to explore the possibility of pFTY720 eliciting beneficial effects directly within the brain during MS. Questioning this possibility is a recent study showing an S1PR1 antagonist, namely Ex26cmpd, does not enter the CNS but causes lymphopenia and ameliorates EAE (Cahalan et al. 2013a). These findings can be consolidated by suggesting that regulation of S1PRs in either the immune system or CNS is capable of attenuating disease.

1.1 S1P metabolism and distribution

Sphingosine 1-phosphate (S1P) is a sphingolipid produced by the metabolism of sphingosine by the sphingosine kinases, sphingosine kinase 1 (SphK 1) and

sphingosine kinase 2 (SphK 2) **(Figure 1.1)**. S1P is found in high concentrations in plasma and lymph due to the production of S1P by erythrocytes and platelets in the blood (Pappu et al. 2007) and by lymphatic endothelial cells (Pham et al. 2010). This production is aided by the presence of large pools of SPHKs and relatively low concentrations of S1P phosphatase, which catabolizes S1P back to sphingosine, and S1P lyase, which terminally cleaves the sphingolipid **(Figure 1.1)**. In contrast, levels of S1P are relatively low in tissues such as the lymphoid organs, likely due to the presence and activity of S1P lyase (Schwab et al. 2005). S1P is mostly bound to high density lipoprotein (HDL) or albumin in the blood and this acts as a ligand reservoir that can act tonicly on extracellular receptors such as those on the endothelial cells of the vascular walls (Murata et al. 2000). This pool of S1P also acts as a control mechanism to prevent over production of the pro-apoptotic precursor ceramide, a key player in the initiation of the apoptotic pathway (Hannun & Obeid 2008).

1.2 S1PR subtypes and expression

S1PRs are a family of G-protein coupled receptors of which there are five subtypes (S1PR1 -S1PR5), each approximately 400 amino acids in length. The genes that code for S1PR2, R4 and R5 are all found on chromosome 19, with S1PR2 and R5 in the same vicinity on 19p13.2, while S1PR1 is found on 1p21 and S1PR3 on 9q22.2 (Rosen et al. 2009). S1P is found in high concentrations (low micro-molar range) in plasma and lymph (Schwab et al. 2005; Pappu et al. 2007; Pham et al. 2010) due to its production by erythrocytes and platelets in the blood (Pappu et al. 2007) and by lymphatic endothelial cells (Pham et al. 2010). Conversely, the interstitial fluid of the lymphoid organ contains low concentrations (nano-molar) of S1P (Schwab & Cyster 2007). The expression of S1PR1, S1PR2 and S1PR3 are widespread, with these subtypes being found in cells of the immune, cardiovascular and central nervous systems (**Table 1.1**). In contrast, reports suggest that S1PR4 is found solely in lymphatic tissue, while the S1PR5 subtype is located primarily on Natural Killer cells (NK cells) (Walzer et al. 2007) and oligodendrocytes (Hla & Brinkmann 2011). In addition to being differentially expressed, the five S1PRs also signal through a multitude of G-protein coupled pathways. In particular, S1PR1 is coupled exclusively to the G_i-pathway (Rosen et al. 2007), S1PR3 and S1PR5 signal through G_i, G_q or $G_{12/13}$ (Jiang et al. 2007) and S1PR2 acts through G_s, G_q or $G_{12/13}$ -pathways (**Figure 1.2**). This repertoire of G-protein coupling by S1PRs allows for signalling via a variety of downstream molecules that include Rac, Rho (Takashima et al. 2008), adenylate cyclase upregulation (Jiang et al. 2007), and phospholipase C activation and intracellular calcium mobilization (Im et al. 2004; Björklund et al. 2005). Through these

pathways, S1PRs are involved in modulating a variety of cellular processes including lymphocyte egress (Mandala et al. 2002), endothelial barrier function (Sanchez et al. 2003), cardiomyocyte regulation (Takuwa et al. 2009), astrocyte migration (Mullershausen et al. 2007) and oligodendrocyte differentiation and survival (Bieberich 2011; Miron, Jung, et al. 2008)

1.3 'Functional antagonism' of S1PR1s

The 'pro-drug' FTY720 is a structural analogue of sphingosine and was found to be phosphorylated *in vitro* (Mandala et al. 2002; Brinkmann et al. 2002) by sphingosine kinases. While S1P is phosphorylated by both SphKs (Pappu et al. 2007), FTY720 appears to be preferentially phosphorylated by SphK2 (Sanchez et al. 2003; Zemann et al. 2006) rather than SphK1 (Sanchez et al. 2003; Allende et al. 2004; Michaud et al. 2006). The phosphorylated form of FTY720 (pFTY720) is a high affinity agonist for S1PR1, S1PR4 and S1PR5, as well as being a partial agonist for the S1PR3, while displaying no affinity toward S1PR2 (Mandala et al. 2002; Brinkmann et al. 2002). Upon binding to the S1PR1 subtype, pFTY720 is thought to induce receptor signalling through several intracellular pathways such as ERK and Ca^{2+} (Healy et al. 2013) and thereafter S1PR1 internalisation, leaving the receptor unavailable for binding with the endogenous ligand, S1P (Gräler & Goetzl 2004) **(Figure 1.3)**. This S1PR1 internalisation induced by pFTY720 has been reported as long lasting, resulting in receptor degradation (Oo et al. 2011; Oo et al. 2007) **(Figure 1.3)**. Thus, pFTY720 is proposed not to act as a classical agonist, but instead has been described as a 'functional antagonist' of S1PR1s **(Figure 1.3)**. This 'functional antagonism' of S1PR1 in lymphocytes is proposed to weaken S1PR1-dependent inhibition of retention signals that are mediated by the chemokine receptor CCR7 (Pham et al. 2008; Gräler & Goetzl 2004; Lo et al. 2005).

The switch from S1PR1-mediated egress to CCR7-mediated retention limits the movement of T cells from lymph nodes and has been suggested as a mechanism explaining the efficacy of pFTY720 in MS (Pham et al. 2008; Gräler & Goetzl 2004; Lo et al. 2005). These effects of pFTY720 likely occur via the S1PR1 subtype, as selective S1PR1 agonists, such as SEW2871, can induce lymphocyte sequestration similar to pFTY720 (Sanna et al. 2004). Further evidence that S1PR1 internalisation is required for lymphopenia comes from studies showing this process is attenuated somewhat in lymphocytes expressing internalisation deficient S1PR1s (Thangada et al. 2010). Additional demonstration of the 'functional antagonist' hypothesis comes from use of specific S1PR1 antagonists such as W146 (Tarrasón et al. 2011),

TASP0251078 (Fujii et al. 2012), TASP0277308 (Fujii et al. 2012), NIBR-0213 (Quancard et al. 2012), and Ex26cmpd (Cahalan et al. 2013b), which induce lymphopenia similar to pFTY720 (Angst et al. 2012). In addition, knockout of S1PR1s from hematopoietic cells is also found to inhibit lymphocyte egress (Matloubian et al. 2004; Allende & Proia 2002). In contrast to antagonism of S1PR1s, T cells overexpressing S1PR1s show reduced homing to lymph nodes and are found at higher levels in blood (Gräler et al. 2005).

1.4 'Persistent signalling' by internalised S1PR1s

An alternative to the 'functional antagonist' theory is provided by a study showing that activation of S1PR1s, by the S1PR1 selective agonist SEW2871 induces receptor internalisation and recycling but can still prevent lymphocyte egress without causing receptor degradation (Jo et al. 2005). Moreover, studies have shown that S1PR1 neutralising antibodies (Visentin et al. 2006) and S1PR1 antagonists, such as W146 (Wei et al. 2005; Sanna et al. 2006), VPC23019 (Wei et al. 2005; Davis et al. 2005) or VPC44116 (Awad et al. 2006; Foss Jr. et al. 2007) do not induce lymphocyte depletion and rather that S1PR1 antagonism can prevent lymphocyte sequestration induced by S1P1 agonism (Foss Jr. et al. 2007). Notably, these earlier findings may be explained by incomplete antagonism of S1PR1's due to insufficient concentrations used in these studies (Tarrasón et al. 2011; Cahalan et al. 2013b). Importantly, however, there is also evidence to suggest that despite S1PR1 internalisation induced by pFTY720, the receptor can 'persistently signal' intracellularly (Mullershausen et al. 2009) and that pFTY720 may be a 'pathway specific modulator' of S1PR1s (Healy et al. 2013; O'Sullivan & Dev 2013) **(Figure 1.3)**.

Studies also suggest that T cell receptor activation causes internalisation of S1PR1s to nuclear membranes, where these intracellular receptors are modified in a 'pathway specific' manner (Liao et al. 2007). Specifically, these nuclear S1PR1s are still $G_{i/o}$-coupled but suppress p-ERK (phosphorylated- extracellular-signal-regulated kinase) and p-cJun (likely limiting cellular function), in contrast to cell surface S1PR1s which promote p-ERK and p-cJun (likely promoting cellular function) (Liao et al. 2007). Paradoxically, S1PR1 antagonism has been shown to increase S1PR1 expression in T cells within the lymph node, which likely has no functional consequence on 'surface expressed' S1PR1s in the presence of continued antagonist application (Cahalan et al. 2013a). It is feasible however that enhanced levels of 'intracellular expressed' S1PR1s may be spared from antagonism and have potentially enhanced signalling. Thus, S1PR1 antagonists, like pFTY720, may work to 'switch' S1PR1 signalling from 'surface

expressed' S1PR1s to 'intracellular expressed' S1PR1s. Use of S1PR1 antagonists in the complete absence of S1P, namely in SphK1/2 double knockout, may help answer if the S1PR1 antagonist-mediated increase in S1PR1 expression plays a role. Thus, there remains considerable debate whether internalisation of S1PR1s causes signalling blockade *per se* or a differential coupling to intracellular signalling pathways (Mullershausen et al. 2009; Healy et al. 2013; O'Sullivan & Dev 2013; Liao et al. 2007).

1.5 S1PR functional antagonism versus persistent signalling

The 'functional antagonism' versus the 'pathway specific persistent signalling' properties of internalised S1PR1s as well as the cycling to and from the cell surface and/or degradation of S1PR1s has been suggested to be dependent on agonist type and concentration of the drug (Rosen et al. 2009) (**Figure 1.3**). Low concentrations of S1PR compounds may induce submaximal receptor occupancy that allows an intracellular pool of receptors to replace those that have been internalised from the cell surface (Rosen et al. 2009; Means et al. 2008). This submaximal occupancy of receptors may also limit the opportunity for modifications that are required to attenuate receptor signalling (Rosen et al. 2009).

In contrast, high concentrations of S1PR ligands may induce S1PR1 internalisation to the endosome and subsequent delivery of the receptor to lysosomes for degradation (Rosen et al. 2009) (**Figure 1.3**). With regard to the type of agonist used, S1P and SEW2871 bound S1PRs are proposed to both follow a 'physiological' receptor cycling pathway whereby they enter endosomes and are recycled back to the membrane (Rosen et al. 2009). In contrast, the binding of pFTY720 to S1PR1s induces persistent internalisation of the receptor, which may then be shuttled to lysosomes for degradation (Oo et al. 2011; Oo et al. 2007) but may also continue to signal internally (Mullershausen et al. 2009; Healy et al. 2013) (**Figure 1.3**). In this regard a supraphysiological activation of S1PR1s by pFTY720 may induce alternative signalling pathways compared to S1P, rather than acting as a functional antagonist (Rosen et al. 2009).

At present, however, the potentially differing mechanisms that are involved in S1P- versus pFTY720-induced internalisation of S1PR1s are not fully known. In addition, the physiologically distinctive roles of surface expressed S1PR1s versus internalised S1PR1s also remain unclear. For example, while S1PR1 activation prevents vascular endothelial growth factor (VEGF)-induced leakage of blood vessels (Sanchez et al. 2003), the antagonism of S1PRs can allow plasma proteins to penetrate into the

tissues of kidneys, lungs and skin (Awad et al. 2006). Moreover S1P signalling, acting through S1PR3s can regulate the functions of cardiomyocites to prevent tachychardia, however, if the plasma level of S1P increases, these receptors become saturated leading to bradychardia (Forrest et al. 2004). Understanding the differences between agonism of surface expressed S1PR1s, antagonism of internalised S1PR1s, or persistent signalling of internalised S1PR1s may thus aid in explaining the physiological role of these receptors.

System	Cell Type	Relative Expression	Source
IMMUNE	T CELL	S1PR1>SIPR4>S1PR2>S1PR3≈S1PR5*	(Graeler & Goetzl, 2002)
	B CELL	S1PR1>S1PR4≥S1PR3**	(Cinamon et al. 2004)
	NK CELL	S1PR5>S1PR4>S1PR1>S1PR2	(Walzer et al. 2007)
	MACROPHAGE	S1PR1≈S1PR2	(Hughes et al. 2008)
CNS	NEURON	S1PR3>S1PR1≈S1PR2>S1PR5≈S1PR4	(Kays et al. 2012)
	ASTROCYTE	S1PR3>S1PR1>S1PR2>S1PR5	(Rao et al. 2003)
	MICROGLIA	S1PR1>S1PR3>S1PR2>S1PR5	(Tham et al. 2003)
	OLIGODENDROCYTE	S1PR5>S1PR1=S1PR2>S1PR3	(Yu et al. 2004)

* CD4 and CD8+ cells express similar patterns of S1PR expression. **Marginal B cells express higher levels of S1PR3 compared to S1PR4. Relative expression shown relates to more common follicular B cells.

Table 1.1 Relative expression of S1PRs in various cell types.

S1PRs are expressed on various tissues but the expression of these receptors on immune cells and within the CNS are, at present, of the greatest clinical significance. This table displays the relative prevalence of the five S1PR subtypes on the main cells of the immune and central nervous systems.

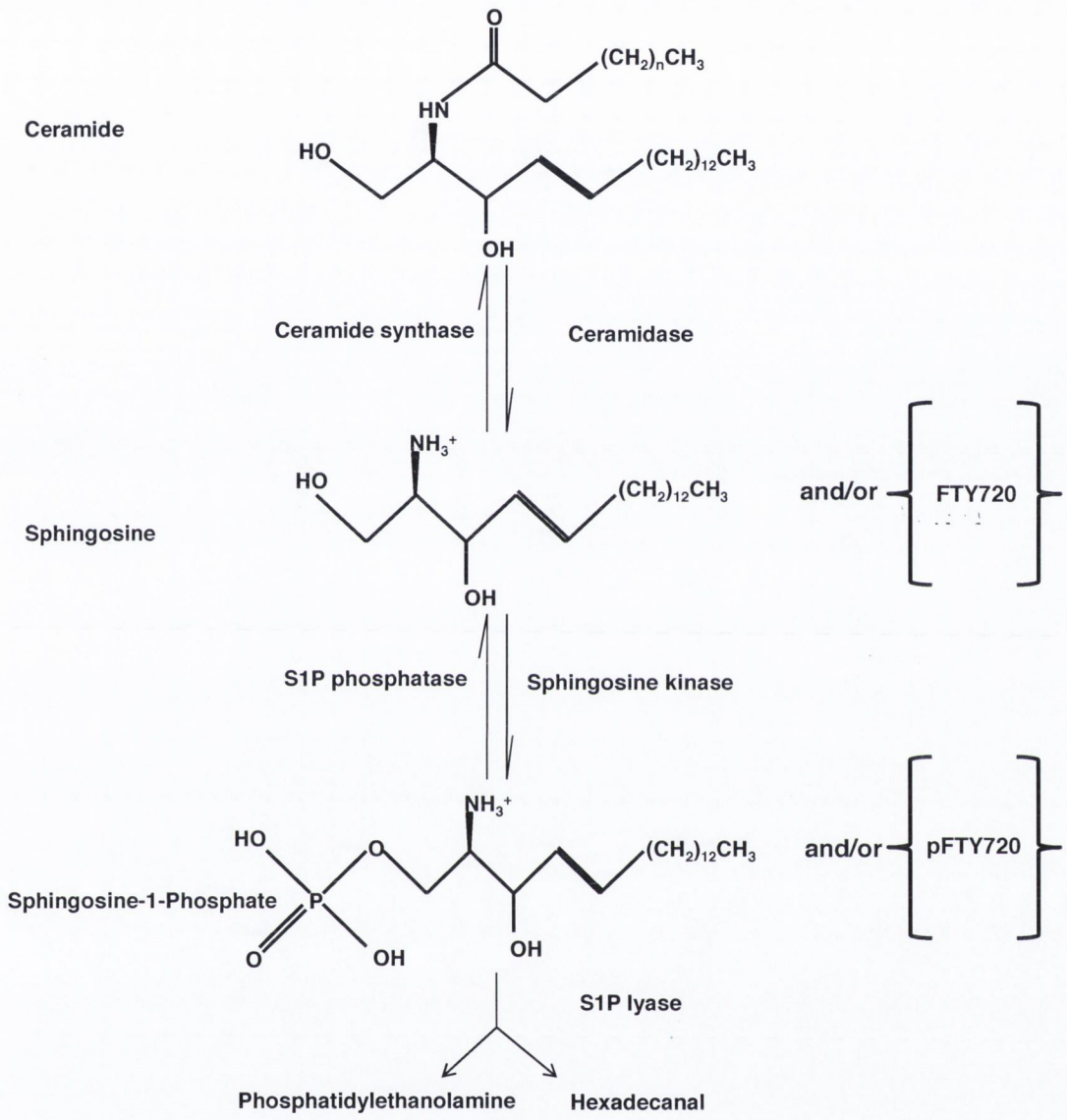

Figure 1.1 Schematic of the S1P synthesis pathway.

Sphingosine is synthesised from ceramide by ceramidase. Sphingosine is in turn phosphorylated to sphingosine-1 phosphate (S1P) by one of two sphingosine kinases (sphingosine kinase 1/2, SphK1/2). S1P lyase then metabolises S1P into one of two products, phosphatidylethanolamine or hexadecanal. FTY720 is also phosphorylated by SphK 1/2 while pFTY720 is dephosphorylated by S1P phosphatases.

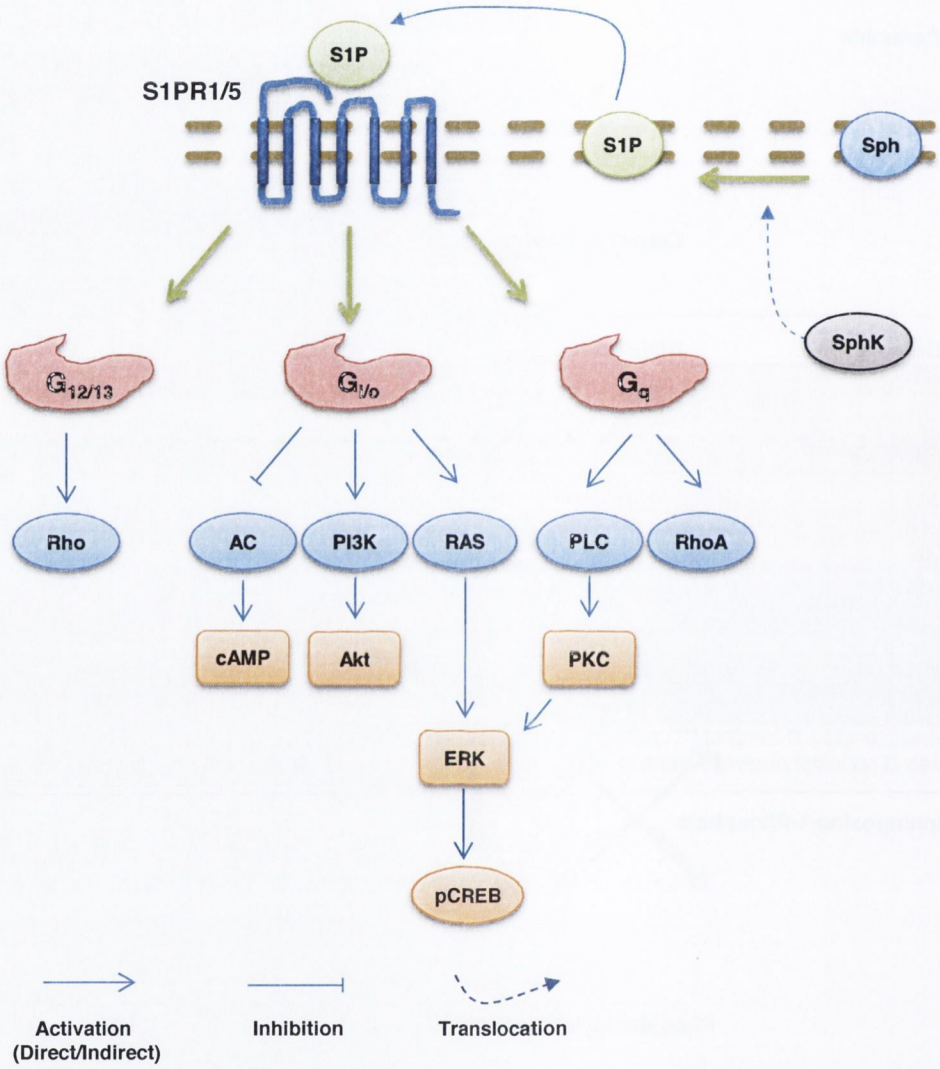

Figure 1.2 Signalling pathways activated downstream of S1PRs

S1PR1 is coupled to the $G\alpha_i$ pathway while S1PR5 primarily acts through the $G\alpha_q$ or $G\alpha_{12/13}$ pathways. In this way, one ligand is able to activate numerous signaling cascades depending on temporal or spatial variations. This preferential coupling is conserved across cell types. [AC, Adenylate Cyclase; PLC, Phospholipase C; cAMP, cyclic adenosine monophosphate; Pi3K, Phosphoinositide 3-kinase; PKC, Protein Kinase C; Sph, sphingosine; SphK1, Sphingosine Kinase 1; ERK, Extracellular signal-regulated kinase; RhoA, Ras homolog A; CREB, cAMP response element-binding protein]

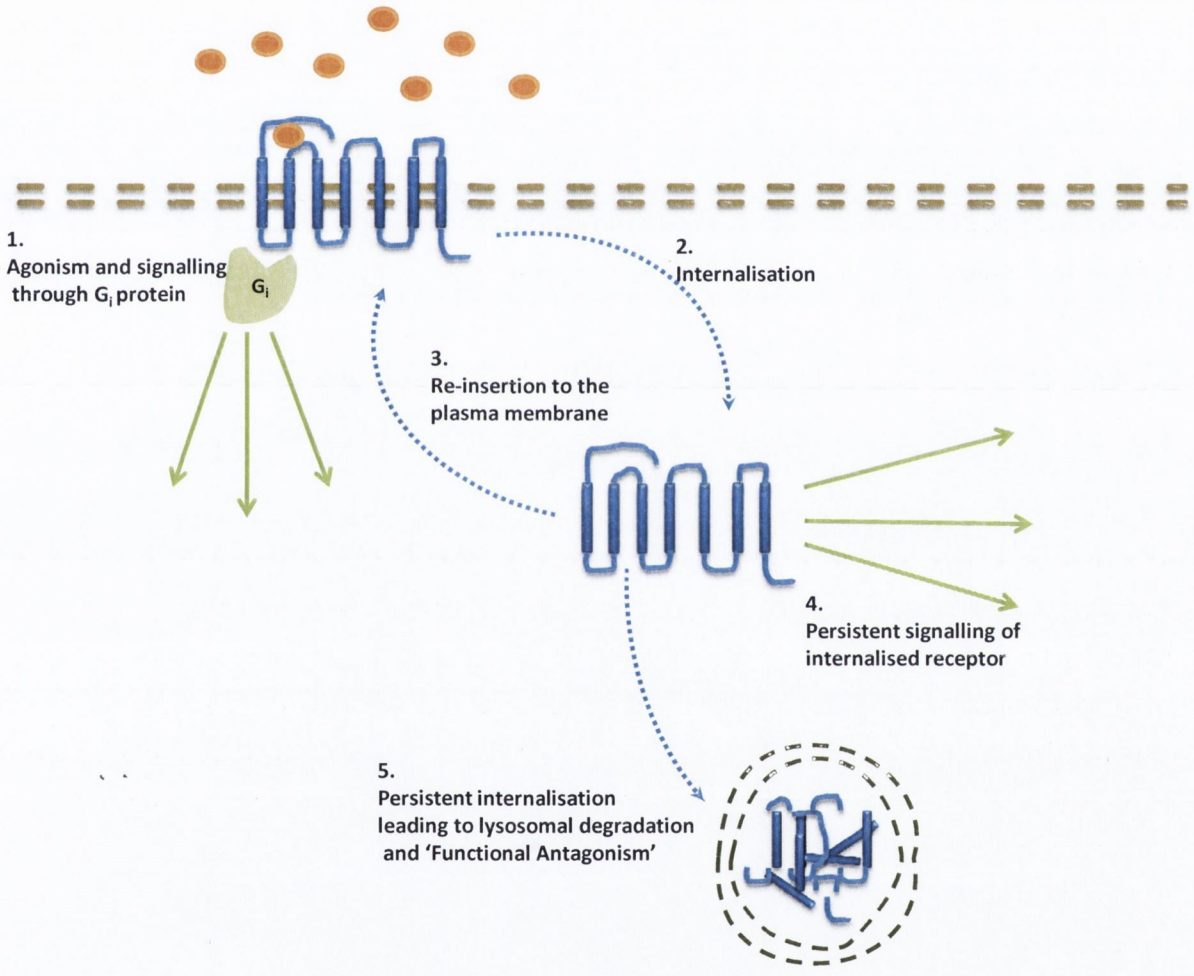

Figure 1.3 S1PR1 cycling pathways

The fate of S1PR1 upon ligand binding is dependent on the type and concentration of agonist. **(1)** S1PR1 signals through a G_i-coupled mechanism to activate a variety of downstream signalling molecules. **(2)** The receptor is internalised and **(3)** may be quickly recycled back to the plasma membrane. **(4)** Agonism by pFTY720 induces internalisation accompanied by persistent signalling of the receptor. **(5)** Persistent internalisation leads to lysosomal degradation and 'functional antagonism'. The mechanisms governing internalisation, recycling and persistent signalling are not fully understood.

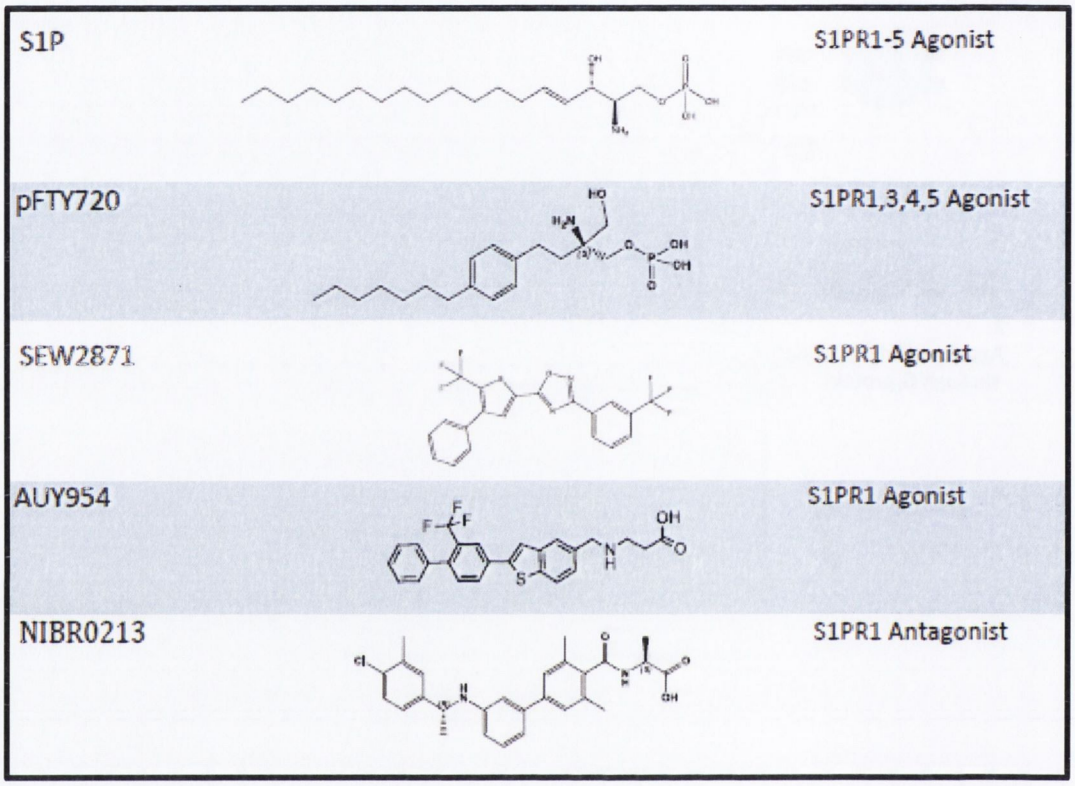

Figure 1.4 Selected S1PR agonists and antagonists

Since the advent of the investigation of S1PR as druggable targets, numerous specific agonists have been developed for both *in vitro* and therapeutic use. In addition to these agonists, antagonists have also been developed. The structures of the primary agonists and antagonists described in this document are displayed above.

2. S1PR1 regulation of immunity

One of the functions of S1P receptors that is of a clinical interest is the regulation of lymphocyte egress from secondary lymphoid organs. S1P release into lymph is regulated by lymphatic endothelium whereas S1P found in plasma is thought to predominantly come from erythrocytes and platelets (Pappu et al. 2007). The internal tissue of the lymph node (LN) has a relatively low level of S1P expression; however, the circulating lymph beyond the sinus-lining endothelia displays a comparatively higher level of S1P. Lymphocytes exit the LN according to this positive S1P concentration gradient, pass into the efferent lymph and hence into the blood-stream (Matloubian et al. 2004). Expression of S1PR1 is also important because upon activation by antigen in peripheral LN, S1PR1 surface expression is temporarily down-regulated on lymphocytes ensuring that they are retained within the organ (Lo et al., 2005). Shortly after activation the receptor is expressed once more and cells become sensitive to S1P again, allowing them to exit the LN. This regulation of receptor expression allows the cells to proliferate and differentiate before returning to the circulation and trafficking to the site of infection (Lo et al., 2005) **(Figure 1.5)**.

2.1 Differential sequestration of T cell subsets

Multiple Sclerosis (MS) is a neurological disease that is believed to have an autoimmune aetiology. MS is characterised by periods of inflammation and demyelination within the CNS and it is thought that this inflammation and demyelination is due to the passage of autoreactive immune cells from the periphery, across the blood-brain barrier and into the CNS. Once these cells find themselves within the brain, they become re-activated against myelin antigens and attack the axonal myelin-sheath (Compston & Coles 2008). Repeated episodes of inflammation and demyelination cause increasing and, ultimately, severe disability in the patient (Compston & Coles 2008). Therapies such as pFTY720 or natalizumab, which prevent the entry of these immune cells into the brain, thereby reducing inflammation and myelin injury, have been shown to be effective treatments for MS (Kappos et al. 2010; Miller et al. 2003). Furthermore, a direct comparison between interferon-β1 and FTY720 suggested that FTY720 treated patients displayed a reduced relapse rate as well as a better MRI outcome than patients treated with interferon-β1 (Cohen et al. 2010). Based on the efficacy of FTY720, S1PRs have been adopted as promising novel drug targets for the treatment of MS. It has been established that the trafficking of some T cell subsets is less affected by FTY720/S1PR1 signalling than others. It is primarily central memory T cells (Tcm) and naive T cells (Tn) that are sequestered in

the LN by FTY720, while effector memory T cells (Tem) can pass into the periphery unobstructed. Tcm and Tn cells express the chemokine receptor CCR7 while long-lived, terminally differentiated Tem do not express this receptor. The hypothesis is that signalling through the S1PR1 overrides the chemotactic effects of signalling through CCR7 and allows the cells to egress (Mehling et al. 2008). It has also been shown that S1P receptor modulation may have effects on T cell proliferation and cytokine release (Wang et al. 2005)

2.2 S1P receptor regulation of T cell function

Wang et al. (2005) showed that S1P acting through S1PR4, decreases T cell proliferation as well as the release of cytokines such as interleukin-4 (IL-4) and IL-2 as well as interferon-γ (IFN-γ) while increasing the release of others, namely IL-10. Regulatory T cells (Tregs) seem to be less affected by S1PR internalisation than some other mature $CD4^+$ T cell types and the proportion of these cells found in the spleen and blood after FTY720 treatment is seen to be increased relative to the numbers seen with no treatment (Sehrawat & Rouse 2008). This may be due to a reduced reliance on S1P gradients to navigate out of lymph node but there is also evidence to suggest that the absolute number of these cells may increase due to the presence of FTY720 (Kim et al. 2011; Daniel et al. 2007; Sehrawat & Rouse 2008). Furthermore, the ability of these cells to bring about their anti-inflammatory and immunomodulatory effects is also seen to be increased after FTY720 treatment (Sawicka et al. 2005). In an animal model of diabetes it has been shown that FTY720 causes an increase in $Foxp3^+$ Treg cells in lymphoid organs in NOD mice. Treatment with the compound induced Foxp3 expression in $Foxp3^-CD4^+$ T cells both *in vitro* and *in vitro* (Sun et al. 2011). The researchers were able to inhibit this effect through the use of a transforming growth factor-β (TGF-β) neutralizing antibody (Sun et al. 2011). This suggests that there may be some crossover between the pathways activated by TGF-β, which is known to increase the expression of Foxp3 in T cells, and the pathways activated by FTY720 upon binding to S1PRs.

The increased regulatory ability of T cells after treatment with FTY720 has also been demonstrated in a mouse model of colitis (Daniel et al. 2007) while additional studies have shown that FTY720 can increase the expression of Foxp3 at both the mRNA and protein level in mixed lymphocyte culture and that it can give these cells the regulatory ability to inhibit the alloreactivity of other lymphocytes (Zhou et al. 2009). However, a seemingly opposing effect has also been reported. One group have shown that FTY720 "potently inhibits regulatory T cell proliferation *in vitro* and *in vitro*" and that

Tregs that undergo adoptive transfer and are treated with FTY720 *ex vivo* lose their suppressive ability (Wolf et al. 2009). The researchers describe how FTY720 inhibits the downstream activation of STAT5 by IL-2 and thereby prevents the normal proliferative response seen upon activation of the IL-2 receptor (Wolf et al. 2009). These studies present opposing descriptions of the effects of FTY720 on Treg proliferation and function. The proliferation and functional enhancement of these CD4+CD25+Foxp3+ regulatory T cells would likely be beneficial to a patient suffering from multiple sclerosis as it would augment the body's own defence against autoimmunity. On the other hand, if FTY720 prevents the proliferation and possibly the functional ability of these cells it could possibly cause an increased susceptibility to an attack by autoreactive cells. FTY720 is also reported to have an anti-inflammatory effect on a subset of T cells, Th17 cells, that have been stimulated with S1P (Liao et al. 2007). IL-23 is considered to be an important instigator of Th17 development and driver of their IL-17 release. According to Liao et al. (2007), S1P can also fulfil this role and brings about a similar if not identical augmentation of Th17 activity. FTY720 was then shown to inhibit the inflammation mediated by these cells thus aiding the immunosuppressive effect of the compound (Liao et al. 2007). Whether this is due to functional antagonism or alternative receptor signalling is not elucidated.

2.3 S1PRs and endothelial cell function

There is direct evidence for a role of the S1P-S1PR system in the regulation of endothelial barrier function. This regulation may contribute to the retention of lymphocytes in lymph nodes by tightening the gap junctions through which these cells exit the lymphoid sinus and enter the efferent lymph (Sanchez et al. 2003; Brinkmann et al. 2004; Singer et al. 2005). In addition, this endothelial cell regulation may also exert beneficial effects in diseases and disease models that are characterised by endothelial cell dysfunction. S1PR1 knockouts (KO) have been shown to be embryonically lethal due to impaired vascular maturation (Liu et al. 2000; Allende & Proia 2002; Allende et al. 2003). Due to this embryonic lethality, the later effects of genetic KO have not been established. Pharmacological tools have therefore been utilised to examine how modulation of S1PR1 affects endothelial cell function. The S1PR1 is expressed on vascular endothelium (Liu et al. 2000) and activation of S1PR1 has been shown to activate both the ERK and Akt pathways (Sanchez et al. 2003). In this way, activation of S1PR1 protects from VEGF induced vasculature leakage in the skin and LPS induced leakage in lung tissue (Sanchez et al. 2003; Brinkmann & Baumruker 2006) S1PR1 is also expressed in lymphatic endothelium (Singer et al. 2005) and may contribute to retention of lymphocytes within lymph nodes. S1P, acting

through S1PR1, induces enhanced organization and redistribution of VE-cadherin and β-catenin in cytoskeletal and junctional complexes in both vascular and sinus-lining lymphatic endothelium (Sanchez et al. 2003; Brinkmann et al. 2004; Singer et al. 2005). Two-photon imaging of lymphocyte egress in the medulla of intact lymph nodes demonstrates that lymphocytes cross into lymphatic sinuses at specific openings, which close following S1P1 agonist stimulation and reopen following restoration of normal receptor tone in the presence of a competing antagonist for S1PR1 (Wei et al. 2005).

In addition to regulating endothelial barrier integrity S1P also regulates endothelial cell migration (Lee et al. 2001). This process requires the Akt-mediated phosphorylation of S1PR1. Activated Akt binds to S1PR1 and phosphorylates the third intracellular loop at the T236 residue. Transactivation of S1PR1 by Akt is not required for Gi-dependent signalling but is indispensable for Rac activation, cortical actin assembly, and chemotaxis (Lee et al. 2001). Whereas the S1PR1 regulates endothelial barrier function by coupling to G_i and Rac1, the signalling pathways involved in the S1P-induced regulation of angiogenesis are not well described. It is known that S1PR1 is necessary for proper maturation of vasculature but the role of other S1PRs has not received the same level of attention.

Recent studies have also shown that while S1PR1 may be involved in growth and angiogenesis, S1PR2 may be more involved in inhibition of growth and induction of proinflammatory responses. In this regard it has been shown that the angiogenic sprouting of human umbilical vein endothelial cells (HUVEC) is suppressed via the S1PR2 subtype likely involving the activation of RhoC via $G_{12/13}$ and Rho specific guanine nucleotide exchange factor leukaemia associated RhoGEF (LARG) (Del Galdo et al. 2013). Furthermore, treatment of endothelial cells with tumour necrosis factor alpha (TNFα) increases the expression of S1PR2. TNFα treatment also enhances SphK1 expression and increases S1P production. Inhibition of the S1PR2 attenuated the TNFα-induced VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) expression in endothelial cells (Zhang et al. 2013). Also, S1P/S1PR2 signalling was shown to stimulate NFκB activation. Moreover, the S1P/S1PR2-stimulated VCAM-1/ICAM-1 expression was completely abolished by inhibition of NFκB. In summary, these studies indicate that TNFα treatment activates autocrine S1P/S1PR2 signalling, which subsequently activates NFκB and leads to the proinflammatory responses in endothelial cells (Zhang et al. 2013).

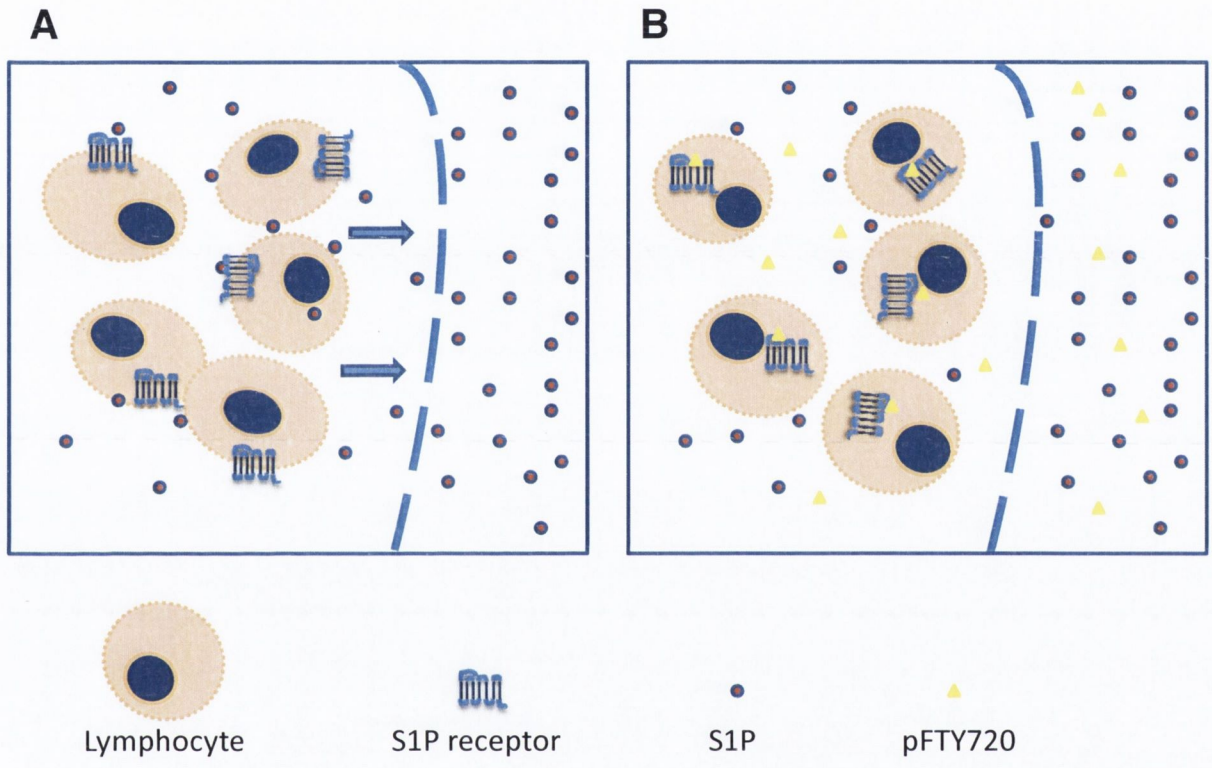

Figure 1.5 pFTY720 prevents T cell egress via 'functional antagonism'

(A) Lymphocytes exit the lymph node according to S1P concentration gradients. They then pass into the efferent lymph and hence into the blood-stream. **(B)** pFTY720 induces persistent internalization of S1PR1 on T cells, leaving them unable to sense this S1P gradient and thus they are retained within the lymph node

Model	Animal strain/species	Reference
EAE	Wistar rat	Brinkmann et al., 2002
Lupus nephritis	NZB/W F1 mouse	Alperovich et al., 2007
Systemic lupus erythematosus	MRL/lpr mouse	Okazaki et al., 2002
Adjuvant-induced arthritis	Rat	Matsuura et al., 2000
Collagen-induced arthritis	Rat	Matsuura et al., 2000
Experimental autoimmune myocarditis	Lewis rat	Kitabayashi et al.,2000
Autoimmune diabetes	CBA mice	Fu et al., 2002
Cerebral ischaemia	Rat	Wei et al., 2010
Transplantation	Rat/mouse/dog	Brinkmann et al., 2001

Table 1.2 Use of FTY720 in animal models

The efficacy of FTY720 has been tested in numerous animal models of disease, particularly those that are believed to have an autoimmune aetiology.

3. S1P receptors and the CNS

In addition to its immunomodulatory effects, FTY720 also interacts with S1P receptors expressed on neurons and glia (Dev et al. 2008). Due to its lipophilic properties FTY720 can easily cross the blood brain barrier and this ability to penetrate the CNS, in combination with the expression of S1PRs on neurons and glia, means that FTY720 activity within the CNS may also contribute to its efficacy as an MS therapy (Dev et al. 2008; Pritchard & Dev 2013). It has been shown that S1P acts through a number of its receptors in the CNS and may regulate the survival, migration and proliferation of oligodendrocytes (Jaillard et al. 2005), astrocytes (Mullershausen et al. 2007) and neurons respectively (Harada et al. 2004). S1PR activation can exert differential effects on cellular processes in the CNS depending on the cell type, developmental stage, receptor subtype and ligand concentration.

3.1 The effects of S1P receptor activation on neurons and their progenitors

There is evidence that functional S1PRs are expressed in areas of active neurogenesis in embryonic rat brain with mRNA transcripts for S1PR1,2,3 and 5 seen in hippocampal neural progenitor cells (NPCs) (Harada et al. 2004). S1P was also shown to induce ERK phosphorylation and cellular proliferation that was sensitive to pertussis toxin (PTx) in NPC cultures (Harada et al. 2004). This indicates that S1PR signalling is involved in the promotion of neurogenesis and operates through a $G_{i/o}$-coupled GPCR, likely S1PR1. S1P signalling is also indicated in the process of neurite extension. In pheochromocytoma (PC12) cells and dorsal root ganglions, nerve growth factor (NGF) has been shown to translocate SphK1 to the plasma membrane, increasing S1P concentration and activating S1PR1 and S1PR2 in a SphK1 dependent manner (Toman et al. 2004) (**Figure 1.6**). Down-regulation of S1PR1 expression suppresses NGF-induced neurite extension while overexpression and transactivation of S1PR1 by NGF enhances neurite extension and stimulation of small GTPase RAC. NGF signalling down-regulates S1PR2, whose activation activates Rho and inhibits neurite extension (Toman et al. 2004). However, prolonged exposure of mature hippocampal neurons to high concentrations of S1P (10μM) releases intracellular calcium and induces apoptosis, likely by activating calcineurin and other phosphatase cascades (Moore et al. 1999).

It is unlikely that S1P in the brain would ever reach these levels but the effect of calcium release and apparent similarity to glutamate-induced excitotoxicity is

interesting. Indeed, S1P signalling has been shown to inhibit glutamate neurotransmission as measured by electrophysiological recordings in cortical neurons *in vitro* (Sim-Selley et al. 2009). This effect was mimicked by the S1PR1 specific agonist SEW2871 and inhibited by VPC44116, an S1PR1/3 agonist indicating that inhibition was dependent on $S1P_1$ signalling but overridden by S1PR3 activation. Behavioural effects such as thermal antinociception, hypothermia, catalepsy and hypolocomotion were associated with this glutamatergic inhibition (Sim-Selley et al. 2009). It may be the case that S1P induced Ca^{2+} release depletes the intracellular stores available for glutamatergic transmission thereby reducing neuronal excitation. In addition, S1P has been shown to induce glutamate secretion from the presynapse in hippocampal neurons as well as potentiating depolarization evoked glutamate release (Kajimoto et al. 2007). The apparent disparity between these reported effects of S1P on glutamatergic transmission are possibly concentration dependent with maximal glutamate secretion induced by 10nM S1P (EC_{50}~ 20pM) whereas glutamate neurotransmission was inhibited with a 1μM dose (Kajimoto et al. 2007; Sim-Selley et al. 2009)

3.2 The effects of S1P receptor activation on astrocyte migration and function

Activation of S1PRs on astrocytes leads to various intracellular effects, including an influx of calcium, phosphoinositide hydrolysis, phosphorylation of ERK and release of [3H]-arachidonic acid (AA) (Rao et al. 2003). These signalling events downstream of receptor activation were inhibited to varying degrees by PTX pre-treatment or by the inhibition of sphingosine kinase. This indicates S1P signalling on astrocytes involves both G_i and G_q-coupled GPCRs (Rao et al. 2003). S1P is released by cerebellar astrocytes in response to basic fibroblast growth factor (bFGF) and induces astrocyte proliferation through G_i-protein-coupled S1PRs (Bassi et al. 2006). This effect is likely beneficial in decreasing BBB permeability as both bFGF and S1P are involved in endothelial tight junction maintenance and astrocyte growth (Abbott et al. 2006). S1P-treated astrocytes are also able to induce neuronal differentiation of NPCs by increasing the levels of laminin (Spohr et al. 2012) however not all effects of S1P activation on astrocytes may be beneficial. Fischer et al. (2011) observed that S1PR3 and SphK1 are upregulated in MS lesions and consequently demonstrated that the SphK1/S1PR3 signalling axis is up-regulated when astrocytes are activated by lipopolysaccharides (LPS), mimicking an inflammatory insult (Fischer et al. 2011). This study also showed increased ERK phosphorylation in response to a selective S1PR3

agonist, similar to findings previously seen with both pFTY720 and a selective S1PR1 agonist (Osinde et al. 2007). Increased astrocyte migration due to pFTY720 is largely via S1PR1 activation (Mullershausen et al. 2007) whereas S1P activates both S1PR1 and S1PR3 (Fischer et al. 2011; Osinde et al. 2007). This signalling pathway appears to play a role in the establishment and maintenance of astrocyte activation, however, increased astrocyte activation and migration could be detrimental in MS due to increased astrogliosis. On the other hand, there may be beneficial effects due to CXCL1 (GROα) release, a neuroprotective cytokine (Fischer et al. 2011). Indeed, it seems that the beneficial effects of FTY720 treatment in MS may be reliant on astrocytic activation. FTY720 efficacy is lost in CNS mutants lacking S1PR1 on GFAP-expressing astrocytes but not on neurons despite the expected lymphopenia due to FTY720 treatment (Choi et al. 2011). It was confirmed via *in situ* hybridization studies that astrocyte loss of S1PR1 is the key alteration in functionally affected mutants (Choi et al. 2011).

3.3 S1PR signalling in oligodendrocytes and their precursors

The first report of S1PRs playing a role in oligodendrocytes came from a study using the oligodendrocyte cell line CEINGE cl3, where treatment with S1P stimulated calcium signalling via a platelet derived growth factor (PDGF) linked mechanism (Fatatis & Miller 1996). Since then, studies have shown human oligodendrocytes express S1PR transcripts in the relative abundance of S1PR5> S1PR1=S1PR2>S1PR3, with undetectable levels of S1PR4 (Yu et al. 2004). Given that pFTY720 does not modulate S1PR2 and that S1PR4 is not reported to be expressed in cells of neuronal lineage, the role of S1PR1, R3 and R5 in the CNS have been particularly studied. Not surprisingly, therefore, S1PR compounds have been reported to regulate a number of intracellular signalling pathways that are linked to S1PR1, R3 and R5 in oligodendrocytes. As indicated previously, S1PR1 is coupled exclusively to the G_i pathway (Rosen et al. 2007) while S1PR3 and S1PR5 signal through G_i, G_q or $G_{12/13}$ (Jiang et al. 2007) (**Figure 1.2**). In oligodendrocytes, S1PRs have been reported to regulate signalling molecules such as adenylate cyclase (AC), phospholipase C (PLC), protein kinase C (PKC), extracellular signal-regulated kinase 2 (ERK2), cAMP-response element binding protein (CREB), phosphoinositide 3-kinase (Pi3K), pAkt and Ras homolog A (RhoA) (Yu et al. 2004; Hida et al. 1999; Saini et al. 2005) (**Figure 1.2**).

S1P and S1PR5 play a role in the regulation of oligodendrocyte precursor cell (OPC) migration. S1P binding to the S1PR5, blocks OPC migration and the use of siRNA to

knockdown S1PR5 reverses this blockade (Novgorodov et al. 2007). This modulation of OPC migration is insensitive to pertussis toxin, suggesting that S1PR5-initiated signaling is not mediated by the G_i-protein coupled pathway and S1PR5 appears to engage the $G_{12/13}$ protein coupled Rho/ROCK signaling pathway to impede OPC migration (Novgorodov et al. 2007). Furthermore, S1P activation of S1PR5 on O4-positive pre-oligodendrocytes induces process retraction via a Rho kinase/collapsin response-mediated protein signaling pathway, whereas no retraction is elicited by S1P on these cells derived from S1PR5-deficient mice (Jaillard et al. 2005). This S1PR5-mediated process retraction is restricted to immature cells and is not observed at later developmental stages. In contrast, S1P activation promotes the survival of mature oligodendrocytes but not of progenitors. This survival mechanism is pertussis toxin-sensitive indicating G_i-coupled GPCR activation and is reliant on Akt phosphorylation (Jaillard et al. 2005).

S1P induces a preferential activation of extracellular signal-regulated kinase 2 (ERK2) in oligodendrocytes (Hida et al. 1999). This S1P receptor activation involves receptor coupling to heterotrimeric G_q subunits with consequent activation of PLC, PKC, and mitogen activated protein kinase (MAPK) pathways leading to ERK phosphorylation (Yu et al. 2004). Studies by Jung et al. (2007) demonstrated that pFTY720 improves the survival of neonatal rat oligodendrocytes during serum withdrawal, which is associated with the phosphorylation of ERK1/2 and Akt. pFTY720 also regulates OPC differentiation into mature oligodendrocytes in a concentration-dependent manner; and S1PRs are differentially modulated by platelet-derived growth factor (PDGF) resulting in downregulation of S1PR5 and upregulation of S1PR1 in OPCs. This in turn promotes PDGF-induced OPC mitogenesis (Jung et al. 2007). The effects of S1PR activation on OPC activity are not just dependent on the ligand or receptor subtype but also on whether activation is acute or chronic. Miron et al. (2008) showed that short-term (1 day) pFTY720 treatment causes initial process retraction in OPCs. Retraction was attributed to S1PR3 and S1PR5 activation and downstream Rho signalling. In addition, pFTY720 treatment was also associated with inhibition of OPC differentiation into more mature phenotypes (Miron, et al. 2008). However, continued pFTY720 treatment (2 days) induced process extension and enhanced cell survival associated with increased ERK1/2 phosphorylation attributed to S1PR1 activation (Miron, et al. 2008). Quantitative real-time polymerase chain reaction showed that pFTY720 induced reciprocal and cyclic modulation of S1PR1 and S1PR5 mRNA levels (Miron, et al. 2008).

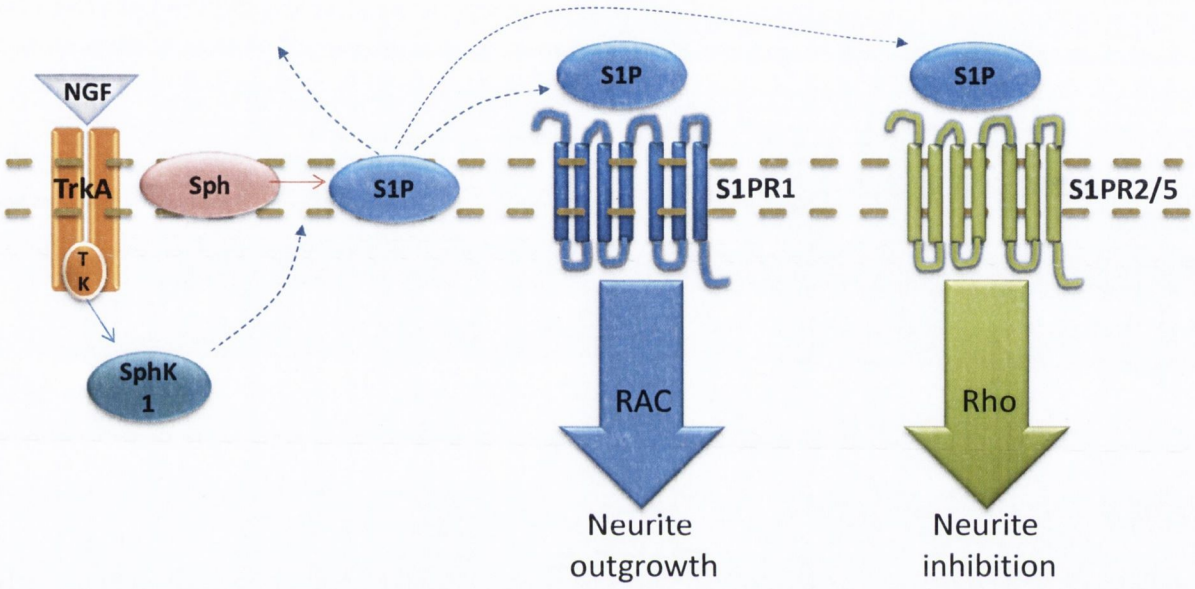

Figure 1.6 Transactivation of S1PR by activation of growth factor receptors on neurons
NGF activation of TrkA type receptor tyrosine kinase induces the translocation of SphK1 to the plasma membrane where it converts sphingosine to S1P. This then acts on extracellular S1PRs to promote neurite outgrowth (through S1PR1) or inhibit neurite outgrowth (S1PR2/5).

4. Relevance of S1PR activation to myelination state

4.1 Cross-talk between S1P, RTK and growth factor receptors.

Growth factors, such as neurotrophin-3 (NT-3) and PDGF, are involved in the repair and regeneration of myelin as well as the differentiation and migration of oligodendrocyte lineage cells (Kumar et al. 1998; Kahn et al. 1999; Kumar et al. 2007). There is much evidence to suggest that growth factors and their receptors may require SphKs in order to propagate signalling events. Furthermore, interactions between growth factor receptors and S1PRs regulate the function of oligodendrocytes and their progenitors thus enhancing myelination state (Saini et al. 2005; Waters et al. 2006; Soliven et al. 2003). The localisation of S1PR1s in caveolae (Igarashi & Michel 2000) brings them into close proximity with the family of receptor tyrosine kinases (RTK) allowing RTK transactivation of S1PR1s (Waters et al. 2006). The transactivation of S1PR1s by RTKs in oligodendrocytes involves, for example, the activation of TrkC receptors by NT-3 that induces a signalling pathway resulting in the translocation of SphK1 from the cytoplasm to the plasma membrane (Saini et al. 2005; Waters et al. 2006). This process is accompanied by increased SphK1 activity in the membrane fraction where its substrate, sphingosine, resides (Saini et al. 2005). SphK1 may then phosphorylate sphingosine to S1P to induce signalling through S1PRs in an autocrine or paracrine manner (**Figure 1.7**). When SphK1 expression is down-regulated by treatment with SphK1 sequence-specific small interfering RNA (siRNA), the capacity of NT-3 to protect oligodendrocyte progenitors from apoptotic cell death induced by growth factor deprivation is attenuated (Saini et al. 2005).

In support of the idea that TrkC receptors transactivate S1PRs, CREB phosphorylation in response to NT-3 involves SphK1 (Saini et al. 2005) (**Figure 1.7**). Moreover, NT-3 can enhance the survival of oligodendrocyte progenitors induced by pFTY720 and also stimulate their maturation. In addition to the transactivation of S1PRs by Trk receptors, growth factors such as PDGF can also regulate S1PRs function by altering receptor expression (Jung et al. 2007). For example, PDGF can differentially downregulate S1PR5 while upregulating S1PR1 in OPCs and thereby promote OPC mitogenesis (Jung et al. 2007). Furthermore, the signalling cascade activated in OPCs in response to PDGF stimulation involves SphK (Soliven et al. 2003). Taken together, the crosstalk between S1PRs, TrkC receptors and PDGF receptors likely play an important role in oligodendrocyte function similar to the cross-talk described between NGF, TrkA and S1PRs that takes place in neurons (**Figure 1.6**).

4.2 S1PRs promote NPC differentiation and survival

It has been shown that S1P and pFTY720 promote the differentiation of NPCs towards an oligodendroglial lineage, as tested by the expression of the OPC markers Olig2 and O4 (Bieberich 2011), as well as promoting the differentiation of NPCs toward the oligodendroglial lineage *in vitro* after transplantation into mouse brain (Bieberich 2011). Moreover, the activation of S1PR1s by S1P or pFTY720 protects NPCs from apoptosis induced by ceramide. In contrast, residual pluripotent stem (rPS) cells do not express S1PR1, which leaves them vulnerable to ceramide induced apoptosis (Bieberich 2011). These data suggest that S1PRs play a role in reducing ceramide-induced apoptosis and promoting the differentiation of NPCs towards the oligodendrocyte lineage. It has also been demonstrated that pFTY720 regulates OPC differentiation into mature oligodendrocytes in a concentration-dependent manner (Jung et al. 2007). In particular, a significant increase in the number of MBP-positive mature oligodendrocytes has been shown using a selective S1PR5 compound (1L), supporting the role of the S1PR5 subtype in oligodendrocyte survival and/or differentiation (Mattes et al. 2010).

4.3 S1PRs modulate oligodendrocyte process extension and retraction

Studies have shown that modulation of S1PRs regulates process extension, oligodendrocyte differentiation and cell survival (Miron, et al. 2008; Jung et al. 2007; Jaillard et al. 2005; Miron, et al. 2008). With regard to process extension and membrane elaboration, a differential role of S1PR1 and S1PR5 in oligodendrocytes has been shown by a number of reports using knockout animals and selective pharmacological tools (Miron, Jung, et al. 2008; Jaillard et al. 2005; Coelho et al. 2007). These effects appear to be dependent upon (i) the type of S1PR drug used, the treatment time and the drug concentration, (ii) the developmental stage of the oligodendrocyte cell type being investigated, and (iii) the differential activation and/or expression levels of S1PRs on these cells (Jung et al. 2007; Miron et al. 2008; Miron et al. 2008). For example, it has been shown that pFTY720 inversely modulates the mRNA expression of S1PR1 and S1PR5 thereby regulating the pattern of process retraction and elaboration in a concentration and time-sensitive manner (Miron, Hall, et al. 2008). While complex, a simplified description of these studies is that, S1PR1 activation primarily plays a role in process extension, while S1PR5 activation is linked to process retraction (Miron et al. 2008; Jung et al. 2007; Jaillard et al. 2005; Miron et al. 2008). In accordance with these *in vitro* studies, it has been shown that human cerebrospinal fluid (CSF) induces process retraction of oligodendrocytes in a S1PR-

dependent manner, suggesting that HDL-associated S1P has the capacity to activate S1PRs in oligodendrocytes *in vitro* (Sato et al. 2007).

4.4 S1PRs regulate oligodendrocyte and neuronal survival and migration.

Rat hippocampal neurons and oligodendrocytes both have the ability to metabolize exogenous ceramide to sphingosine and then to S1P (Qin et al. 2010). While low concentrations of S1P can be metabolized to sphingosine, high concentrations and long term treatments with either ceramide or S1P can lead to apoptotic cell death (Qin et al. 2010). It therefore appears that both neurons and oligodendrocytes can protect themselves from sphingolipid-induced cell death. The direct significance of these findings in relation to MS is seen in white matter and plaques from brains of MS patients, where reduced S1P and increased sphingosine and ceramide can be observed (Qin et al. 2010). Supporting the idea that S1PRs play a role in oligodendrocyte survival, pFTY720 rescues mature human oligodendrocytes from serum and glucose deprivation-induced apoptosis (Miron et al. 2008). These pro-survival effects of pFTY720 are also mimicked by an S1PR5 agonist, implying a role for this receptor subtype and the G_i and G_q-associated activation of Akt (Jaillard et al. 2005; Miron et al. 2008) (**Figure 1.8**).

In OPCs, pFTY720 treatment also enhances cell survival and provides protection from apoptosis (Miron, et al. 2008; Jung et al. 2007; Jaillard et al. 2005; Coelho et al. 2007). In this case, S1PR1s are proposed to play a role, where treatment of OPCs with the S1PR1 selective agonist, SEW2871 enhances survival (**Figure 1.8**). These effects are associated with S1PR1-dependent recruitment of G_i-proteins (but not G_q), increased ERK 1/2 and Akt phosphorylation (Miron, et al. 2008; Coelho et al. 2007). In addition to S1PRs regulating oligodendrocyte survival and differentiation, the activation of these receptors also inhibits OPC migration (Novgorodov et al. 2007). Evidence that the S1PR5 subtype plays a role in OPC migration comes from siRNA knock-down studies, where siRNA against S1PR5 prevents an S1P-induced decrease in OPC migration (Novgorodov et al. 2007). This S1PR5-mediated modulation of OPC migration is independent of G_i signalling and appears to engage a $G_{12/13}$ protein coupled Rho/ROCK pathway (Novgorodov et al. 2007).

4.5 S1PRs maintain myelination state *in vitro*

The infiltration of immune cells that are reactive to components of the myelin sheath into the CNS is thought to be causal in both MS and EAE (Compston & Coles 2008).

Due to the effects of S1PR modulation on immune cells, it has been difficult to elucidate the direct effects of pFTY720 on cells of the CNS *in vitro*. In order to overcome this limitation, the effects of S1PR drugs on myelination have been studied in organotypic cultures treated with the demyelinating agent lysophosphatidylcholine (LPC) (Miron et al. 2010; Sheridan & Dev 2012). Treatment with pFTY720 subsequent to LPC-induced demyelination of organotypic cerebellar slices enhances remyelination and process extension by OPCs and mature oligodendrocytes as well as increasing glial fibrillary acidic protein (GFAP), a marker of astrogliosis (Miron et al. 2010). Similarly, following demyelination by LPC in a rat telencephalon reaggregate-spheroid cell culture system, pFTY720 and the S1PR1/5 agonist BAF312, but not AUY954 (S1PR1 specific agonist), significantly augment expression of myelin basic protein (MBP) in the remyelination phase (Jackson et al. 2011). The treatment of organotypic cerebellar slices with pFTY720 or the S1PR1 selective compound SEW2871, has also been shown to attenuate LPC-induced demyelination (Sheridan & Dev 2012). These studies implicate the S1PR3/5 subtypes in promoting remyelination (Miron et al. 2010) and the S1PR1 subtype in attenuating demyelination (Sheridan & Dev 2012), though it is likely the roles of these S1PRs are not mutually exclusive. The effects of S1PR modulation on myelination state potentially involves a reduction in a number of pro-inflammatory factors such as TNF-α, IL-1, microglial ferritin, LIX (CXCL5), MIP-1alpha, and MIP-3alpha (Jackson et al. 2011; Sheridan & Dev 2012). A reduction in signalling molecules such as nitric oxide metabolites and apoptotic effectors caspase 3 and caspase 7 (Jackson et al. 2011) after treatment with S1PR drugs has also been suggested to preserve myelination state. Taken together, this may suggest that the attenuation of inflammatory, chemotactic and apoptotic factors during demyelination creates an environment more conducive to remyelination.

4.6 S1PRs reduce demyelination *in vitro*

The role of S1PRs in regulating myelination (**Figure 1.8**) has been studied *in vitro* using both genetic and pharmacological approaches (Jaillard et al. 2005; Choi et al. 2011). S1PR1 null animals are embryonically lethal, and studies have reported specific ablation of this receptor subtype from specific cells of the CNS. In the case of S1PR1, the knockout of this receptor subtype from astrocytes (but not neurons) attenuates development of EAE and limits the effects of pFTY720 (Choi et al. 2011). Notably, the specific knockout of S1PR1 from oligodendrocytes has been reported to increase sensitivity to demyelination induced by agents such as cuprizone (Kim et al. 2011). In contrast, no developmental abnormalities have been reported for S1PR5-null mice allowing study of this receptor subtype *in vitro*, without the need to generate

conditional knockout animals (Jaillard et al. 2005). These S1PR5-KO animals have no reported deficits in myelination states (Jaillard et al. 2005), potentially questioning the role of S1PR5s in regulating demyelination and/or remyelination in these animal models. Experiments to determine the behaviour of these S1PR5 KO mice in neuroinflammatory models such as EAE or the effects of S1PR5 selective compounds in such animal models have also yet to be reported.

The roles of S1PRs have also been reported in models of demyelination induced by agents such as cuprizone and LPC (Kim et al. 2011; Hu et al. 2011). In these studies, however, FTY720 has not been shown to promote remyelination (Kim et al. 2011; Hu et al. 2011). Nevertheless, there is some evidence to suggest that fingolimod can be effective in the promotion of remyelination. In a Dark Agouti (DA) rat EAE model, treatment with FTY720 after disease onset attenuated the deficits in both visual and sensory evoked potential and reversed paralysis (Balatoni et al. 2007). This correlated with decreased demyelination compared to untreated EAE controls. It was suggested that the normalisation of electrophysiological recordings and reacquisition of motor function is likely indicative of a remyelination response facilitated by FTY720 therapy (Balatoni et al. 2007). In this study, however, the effects of FTY720 on myelination by either directly regulating oligodendrocytes and/or by limiting inflammatory response in the CNS are difficult to discern. Nevertheless, treatment with FTY720 has been shown to attenuate cuprizone-induced damage to oligodendrocytes in the corpus callosum (Kim et al. 2011). When co-administered with cuprizone, FTY720 reduces astrogliosis and microgliosis but when administered 4-9 weeks after cuprizone, FTY720 augments astrogliosis (Kim et al. 2011). Similar to the reported *in vitro* studies (Jackson et al. 2011; Sheridan & Dev 2012), these protective effects of FTY720 are associated with a reduction in inflammatory cytokine and chemokine transcripts in the corpus callosum, particularly IL-1β and CCL2, as well as altered expression of S1PR1 (Kim et al. 2011). It therefore appears that S1PRs expressed by glia are involved in regulating the response to injury but whether direct CNS effects of pFTY720 can promote myelin repair remains to be fully established. S1PR compounds will likely be further developed as MS therapies and may also be used to treat other inflammatory or autoimmune disorders as evidenced by the animal models in which the compound has been tested (**Table 1.2**).

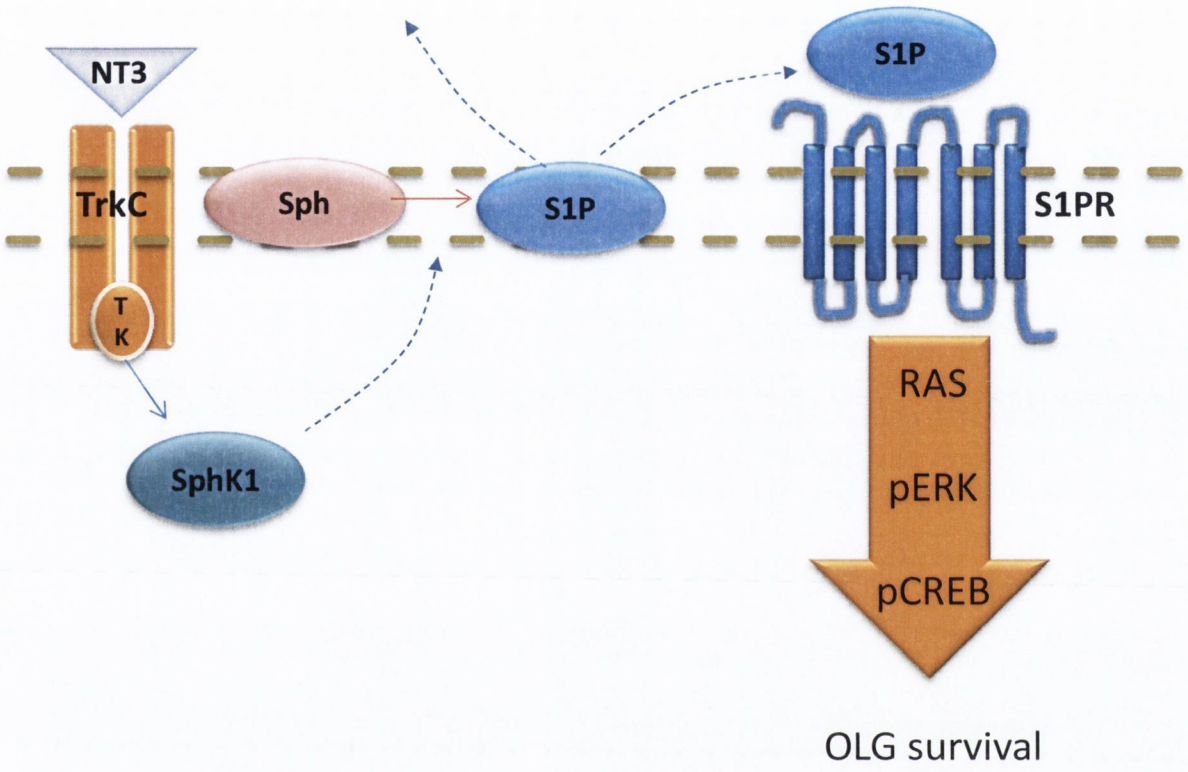

Figure 1.7 Transactivation of S1PR by activation of growth factor receptors on oligodendrocytes

NT3 activation of the receptor tyrosine kinase, TrkC induces the translocation of SphK1 to the plasma membrane where it converts sphingosine to S1P. This then acts on extracellular S1PRs to activate RAS, phosphorylate ERK and consequently CREB, thus promoting oligodendrocyte survival.

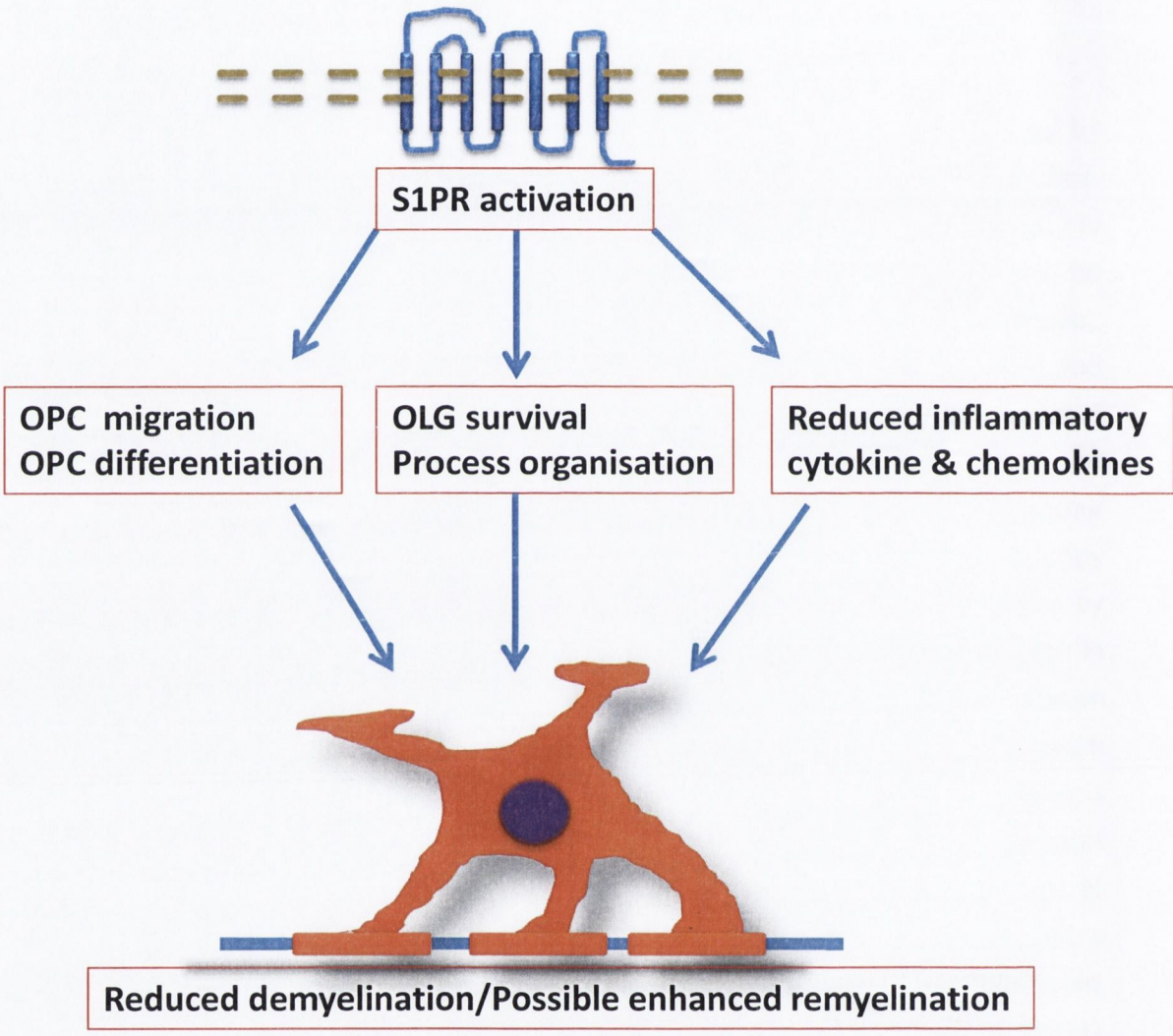

Figure 1.8 Contribution of S1PR modulation to myelination state.

The modulation of S1PRs exerts many effects on oligodendrocytes and their environment that may directly or indirectly contribute to decreased demyelination and possibly enhanced remyelination.

5. Multiple Sclerosis and the role of S1PRs

5.1 Introduction to MS

Multiple Sclerosis was first identified in 1868 by Jean-Martin Charcot (Lublin 2005; Kumar et al. 2011) and since then has become one of the most commonly diagnosed neurological disorders. It is a disease of the central nervous system characterised by inflammation and the demyelination of neuronal axons. Demyelination is centred in various foci and these lesions, or scleroses, of damaged myelin may occur in any region of the brain and spinal cord containing white matter (Compston & Coles 2008), hence the coining of the term 'multiple sclerosis' in 1955 (Compston et al. 2005). This can give rise to a wide variety of neurological deficits including muscle weakness, ataxia, blindness and paralysis as white matter is to be found throughout the brain and spinal cord (Compston & Coles 2008). There are approximately 2.5 million sufferers worldwide (Compston & Coles 2008) and in Ireland the rate of prevalence is somewhere in the region of 0.1-0.2% (McGuigan et al. 2004).

MS can affect people at any age; however, onset usually occurs between the ages of 18 and 50. There is also a higher overall rate of occurrence for women than men, in the ratio of approx. 2:1, although this rate of prevalence is not consistent in all forms of the disease (**Table 1.3**) and may also vary by location. At present, most available therapies are used in the treatment of relapsing remitting MS (RRMS) with few if any effective treatments for the progressive forms currently on the market (**Table 1.4**).

5.2 Current therapeutic options.

The first effective MS therapies were corticosteroid based and while they showed some ability to reduce relapse severity they displayed little or no efficacy in the reduction of relapse rate or disability progression (Lublin 2005). After many immunosuppressive agents were trialed and found to be ineffective, it was discovered that interferonβ-1b (IFNβ-1b) brought about a significant reduction in disease activity and relapse rate in RRMS (The IFNβ Multiple Sclerosis Study Group, 1993). There are now a range of IFNβ based therapies on the market as well as monoclonal antibodies (Natalizumab, Tysabri™, Élan), anti-neoplastics (mitoxantrone) and amino-acid polymer decoys (Glatiramer acetate, Copaxone™, Teva) (**Table 1.4**). Unfortunately, the efficacy and side-effect profile of some of these therapies is undesirable and they all involve self-administered injections or hospitalization for intra-venous infusion. Therefore there has been an unmet clinical need for an effective oral therapy. One such novel therapy, the first oral treatment approved by the FDA for the treatment of

MS, is Fingolimod or FTY720 (Gilenya™, Novartis) (**Table 1.4**). This therapy prevents T cell infiltration into the brain by inducing reversible lymphopenia through its activity on S1PR1s (Brinkmann et al., 2002). FTY720 has been shown to be an effective therapy in clinical trials, with patients displaying fewer lesion, a lower relapse rate and reduced disability when compared to Interferon β-1a (Avonex®, Biogen) treatment, the current front-line therapy (Cohen et al, 2010)

S1PRs have become promising targets in the development of novel MS therapies (Pritchard & Dev 2013) due to the efficacy of FTY720 in both EAE studies as well as clinical trials for MS (Brinkmann et al. 2002; Kappos et al. 2010). It has also been shown that intrathecal levels of S1P are increased in the early stages of MS (Kułakowska et al. 2010) and that the expression of S1PR1 and S1PR3 are upregulated in MS lesions themselves (Van Doorn et al. 2010). These data, combined with the efficacy of FTY720 has led to the development of novel, selective S1PR modulators for the treatment of RRMS and possibly SPMS, such as the S1PR1 and S1PR5 agonist Siponimod (BAF312) (Selmaj et al. 2013; Kappos et al. 2013).

5.3 Aetiology of MS

The cause of MS is still unknown but there is a long-standing theory that both that genetic and environmental factors are involved, however it is more likely that susceptibility to the disease results from an interaction between genetics and environment (Ebers 2013). MS has a familial recurrence rate of about 20%. Overall, the reduction in risk changes from 5% amongst siblings to 1% in second-degree and third-degree relatives. Population-based studies of multiple sclerosis in twins from Canada and the UK show higher clinical concordance rates in monozygotic than in dizygotic pairs (25% vs. 5%) (Compston & Coles 2008).

There is no specific gene mutation associated with MS but rather it is thought to be the result of a range of normal polymorphisms that may each contribute to the overall disease state (Dyment et al. 2004). These genes include, but are not restricted to: HLA classes I and II, T-cell receptor β, *CTLA4*, *ICAM1*, and *SH2D2A* (Dyment et al. 2004). The main MHC haplotype DRB1*1501 is strongly linked to MS susceptibility and this gene is regulated by Vitamin D (Ebers 2013). This has led to the theory that a lack of Vitamin D, synthesised by exposure to sunlight, may play a role in disease aetiology by increasing epigenetic susceptibility to MS through misregulation of genes such as DRB1*1501 (Ebers 2013).

There is also a higher rate of occurrence in developed countries when compared to developing countries. The belief is that the improved levels of sanitation mean that there are fewer immunological insults at an early stage of life thus leading to greater susceptibility to particular infections in young adulthood. Human herpes virus, as well as Epstein-Barr virus, has been indicated as a possible trigger of MS (Farrell et al. 2009; Lünemann et al. 2010) but the direct contribution of viral infection to MS lesions is debatable (Willis et al. 2009).

5.4 Autoimmune activity in MS

Genes of the HLA class II region, specifically the HLA DRB1*1501, DQA1*0102, and DQB1*0602 extended haplotype, as well as the HLA class I region, HLA-A*0301 locus are linked to the major histocompatibility complex (MHC) (Dyment et al. 2004). MHC molecules are involved in the presentation of antigen and consequent activation of cells of the immune system, specifically T cells (Holling et al. 2004). T cells are implicated in the onset and progression of MS even though the mechanism by which they interact with and attack the CNS is not fully established (Compston & Coles 2008). The susceptibility to MS associated with genes of the HLA haplotype and hence MHC class molecule dysfunction (Dyment et al. 2004; Ramagopalan et al. 2009; Ebers 2013), in combination with immune cell activity in both animal models (Murphy et al. 2010) and active MS lesions (Compston & Coles 2008), indicate that it is likely an autoimmune disease. The basic steps that are thought to lead to lesion formation include: BBB breakdown due to inflammation (Minagar & Alexander 2003) infiltration by peripheral immune cells (most importantly T cells, macrophages and B cells); T cell mediated attack on oligodendrocytes/myelin; neuronal conduction impairment and continued attacks leading to permanent damage and even transection of neurons (Bjartmar et al. 2003).

Under normal circumstances the brain is an immunologically privileged organ due to the presence of the BBB (Davson 1976; Zlokovic 2008). The BBB is a specialized endothelial structure that, in conjunction with pericytes, astrocytes, and microglia, separates components of the circulating blood from the brain parenchyma (Zlokovic 2008). In addition, the BBB functions to maintain the composition of the environment, thus allowing for proper activity of neurons (Zlokovic 2008). It is thought that the tight junctions between the endothelial cells of the BBB may become compromised during an infection or viral attack when pro-inflammatory cytokines are released (Minagar & Alexander 2003). There may also be an increase of expression of endothelial cell adhesion molecules (ECAMs) such as ICAM-1, VCAM-1 caused by this cytokine

release (Washington et al. 1994). These molecules allow lymphocytes and macrophages to adhere to and pass along the capillary wall until they reach a point where a junction has been compromised, and then pass through and into the brain (**Figure 1.9).** In MS, these immune cells are auto-reactive and initiate inflammation and demyelination of axons (McFarland & Martin 2007).

5.5 The role of the adaptive immune system in MS

One theory of MS pathogenesis suggests that adaptive immune cells may become autoreactive through a process known as molecular mimicry (Panoutsakopoulou et al. 2001). This theory states that peptides from foreign antigens presented by MHC II molecules can be indistinguishable from self-antigens. This causes the effector cell to become autoreactive to the self-protein, in the case of MS patients, those antigens are contained in myelin (Panoutsakopoulou et al. 2001). Various T cell subtypes may be involved in the onset of MS (**Table 1.5**).

Many of the proposed theories relating to MS aetiology are based on data gathered from the animal model of MS, EAE. In this model, animals, most commonly rodents, are immunised with a component of axonal myelin, such as MBP or myelin oligodendrocyte glycoprotein (MOG) in the presence of an adjuvant and pertussis toxin to increase immune responses. T cells become activated against this antigen and these myelin-reactive cells infiltrate the brain and lead to progressive deficits in motor function due to axonal injury. While EAE is a useful model for the development of MS therapies there are some differences between the disease characteristics in the model when compared to MS. Widespread demyelination is often largely absent in typical EAE models and the demyelination that is seen doesn't occur in the same confluent zones as in MS, especially in MBP-reactive EAE (Gold et al. 2006). There is also a lack of B cell involvement seen in MBP-induced EAE. This B cell activity is now deemed to be involved in MS progression due to the efficacy of B cell targeting compound such as rituximab (Hauser et al. 2008). The MOG induced EAE model does display both T and B cell synergy and is a more faithful reproduction of MS activity than the MBP model (Ray et al. 2011). These standard EAE models also show a total focus on $CD4^+$ cell activity and don't mimic the activity of clonally expanded $CD8^+$ cells (**Table 5**) that are found clustered in the cores of lesions and make up the bulk of the lymphocytic infiltration in patient samples (Skulina et al. 2004). In this regard, new animal models show that myelin-specific $CD8^+$ T cells can also mediate CNS autoimmunity (Goverman 2009).

Th1 cells (**Table 1.5**) have long been considered the main proponents of autoimmunity in MS, however, the activity of a relatively newly identified T cell phenotype, Th17, is now associated with inflammation and axonal lesions (Bettelli et al. 2006) (**Table 1.5**). These cells are named Th17 due to their release of IL-17, a potent inflammatory cytokine (Bettelli et al. 2006). Studies into the abilities of different T cell subsets to induce EAE have found that Th17 cells are effective in inducing EAE and that the Th17 cells activated against MOG cause disease with an overlapping but distinct phenotype when compared to that of Th1 induced EAE (Jäger et al. 2009). Furthermore, it has been shown that Th17 cells producing IL-17 and/or IFNγ infiltrate the CNS prior to the onset of clinical symptoms and are associated with microglial activation. They may also cross the BBB before Th1 cells and therefore play a role in the initial onset of MS (Murphy et al. 2010). Novel research has also suggested that in a MOG induced EAE model, Th17 cells may form direct connections with neurons and mediate axonal damage through what appears to resemble a synaptic connection (Siffrin et al., 2010).

Disease Variant	Characteristics	Prevalence
Relapsing Remitting (RRMS)	Periods of acute symptoms followed by periods of remission. Over time remission shortens and baseline disability increases.	~85% of MS patients are diagnosed with RRMS
Secondary Progressive (SPMS)	Generally follows from RRMS and is characterised by progressive exacerbation of symptoms with no remission phase	50% of patients will present with SPMS within 10 years of RRMS diagnosis
Primary Progressive (PPMS)	Patients display steady decline in function with no remitting phase	~10% of MS patients are diagnosed as having PPMS
Primary Relapsing (PRMS)	Exacerbation of symptoms is followed by remitting phase but there is an overall decline in function	~5% of patients are diagnosed with PRMS
(Andersson & Waubant 1999)		

Table 1.3 Variants and prevalence of MS

MS is not a heterogenous disease but rather presents with different symptoms and follows a varied time-course. Broadly speaking there are deemed to be four main disease variants.

Name	Manufacturer	Description	Indication	Administration	Mechanism	Efficacy
Avonex (interferon beta-1a)	Biogen	Identical amino acid sequence to naturally occurring interferon β-1a	RRMS	Weekly intramuscular injection	Proposed anti-inflammatory properties and may strengthen BBB integrity	Reduces relapse rate by approx. 30% vs. placebo
Rebif (interferon beta-1a)	Ares-Serono	Identical amino acid sequence to naturally occurring interferon β-1a	RRMS	Sub-cutaneous injection 3x per week	Proposed anti-inflammatory properties and may strengthen BBB integrity	Reduces relapse rate by approx. 30% vs. placebo
Betaseron (interferon beta-1b)	Bayer	Identical amino acid sequence to naturally occurring interferon β-1b	RRMS	Sub-cutaneous injection every second day	Proposed anti-inflammatory properties and may strengthen BBB integrity	Reduces relapse rate by up to 40% vs. placebo
Extavia (interferon beta-1b)	Novartis	Identical amino acid sequence to naturally occurring interferon β-1b	RRMS	Sub-cutaneous injection every second day	Proposed anti-inflammatory properties and may strengthen BBB integrity	Reduces relapse rate by up to 40% vs. placebo
Copaxone (glatiramer acetate)	Teva	Random polymer of four amino-acids: Glutamic acid, Lysine, Alanine and Tyrosine	RRMS	Sub-cutaneous injection daily	Unclear. May shift T cells from inflammatory to anti-inflammatory phenotype. May act as decoy for MBP specific T cells	Unclear.
Gilenya (fingolimod)	Novartis	Ligand for S1P receptors	RRMS	Oral capsule daily	Prevents lymphocytes from exiting lymph nodes and entering CNS	Reduces relapse rate by 52% compared to Avonex and also significantly slows disease progression by as much as 30%
Novantrone (mitoxantrone)	Generic	Type II topoisomerase inhibitor	SPMS, PRMS and worsening cases of RRMS	I.V. infusion once every three months	Disrupts DNA synthesis and repair leading to immunosuppression	Can reduce relapse rate by 66% in RRMS. Can slow or partially reverse disability progression based on EDSS*
Tysabri (natalizumab)	Élan/Biogen	Monoclonal antibody for α4-integrin	RRMS	I.V. infusion once every four weeks	Disrupts lymphocyte adhesion through α4-integrin and prevents cells crossing BBB.	Reduces relapse rate by 68% vs. placebo

Table 1.4 Current approved MS therapies and mechanisms of action

Therapies for MS have differing targets and vary in efficacy; however, they mostly aim to prevent symptoms by reducing inflammation and preventing exacerbation of lesions.

Cell	Marker	Promoters	Releases	Function
Cytotoxic	CD8	IL-2	IFN-γ	Reduce viral replication within infected cell and remove pathogen.
Th1	CD4	IFN- γ, IL-12	IL-1, IFN-γ , TNF-α	Detect bacterial antigens and release cytokines that activate macrophages in order to destroy microorganisms. Also induce B cells to produce IgG antibodies to protect against extracellular pathogens.
Th2	CD4	IL-4	IL-10	Release anti-inflammatory IL-10 and induce IgE antibody production by B cells in response to parasitic infection and allergic reactions.
Th17	CD4	IL-23, IL-17	IL-17, IL-6, TNF-α, CXCL1	Neutrophil recruitment to sites of infection.
Treg	CD4, CD25, Foxp3	TGF-β, IL-2	IL-10, TGF-β	Suppression of immune responses of other T cells, including auto-immune responses.

Table 1.5 T cell subtypes and characterization

T cells are a type of mononuclear cell and are one of the primary cell types in the adaptive immune system. There are many members within the T cell family that cooperate to perform different functions in reaction to pathogens from differing sources (Murphy et al., 2008).

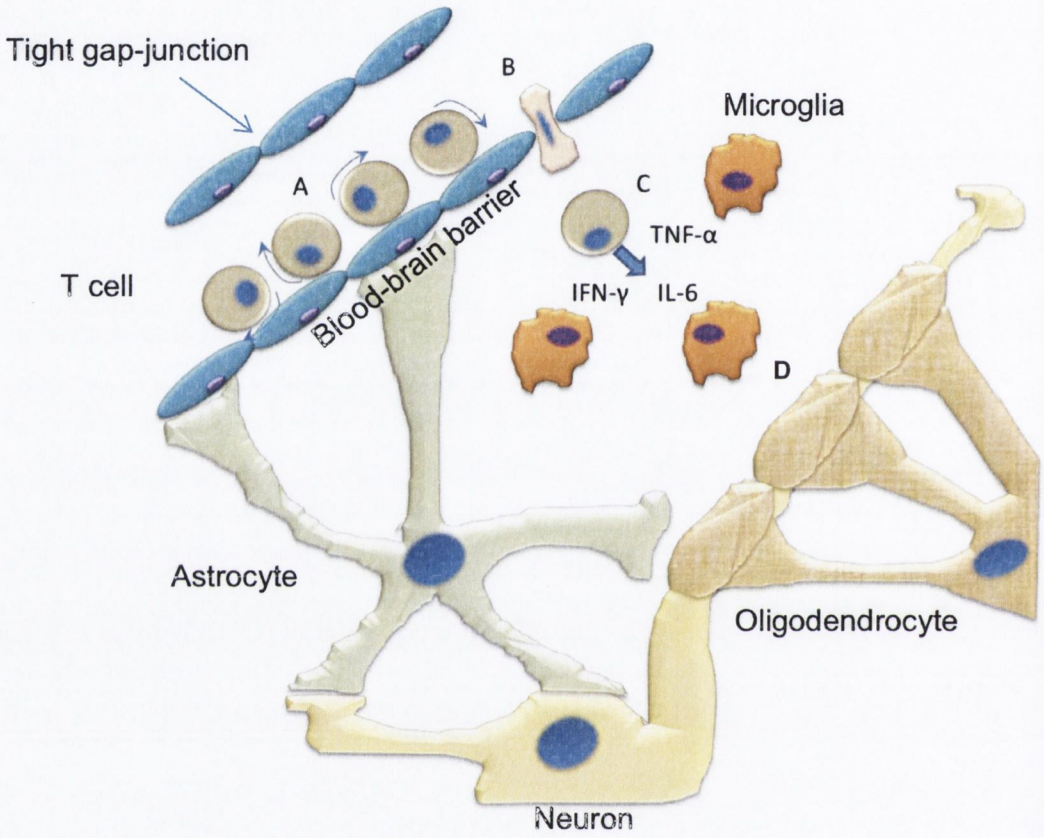

Figure 1.9 T cell passage across the blood-brain barrier.

(A) Activated T cells migrate along the endothelial cell wall until they reach a weakened gap-junction **(B)** possibly due to pre-existing inflammation. The T cells penetrate into the brain parenchyma where they may be re-activated by antigen presenting cells (APCs). They release inflammatory cytokines **(C)** which, in turn, leads to the recruitment and activation of microglia/macrophages that attack the oligodendrocytes that create the myelin sheath enveloping neuronal axons **(D).**

Chapter 2. Materials and Methods

1.Materials

1.1 Chemicals

The following chemicals and reagents were used: Acetic acid (45754-500ml, Sigma, St. Louis, MO), Ammonium persulfate (APS) (A3678-25G, Sigma, St. Louis, MO), ε-amino caproic acid (A7824-25G, Sigma, St. Louis, MO), Bovine serum albumin (BSA) (A3156-5G, Sigma, St. Louis, MO), Dimethyl sulfoxide (DMSO) (D2650-100ML, Sigma, St. Louis, MO), ethylene glycol tetraacetic acid (EGTA) (E3889-25G, Sigma, St. Louis, MO), Ethanol (E7023-500ML, Sigma, St. Louis, MO), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (H3784-100G, Sigma, St. Louis, MO), β-mercaptoethanol (31350-010, Gibco, Carlsbad, CA), Methanol (34966-2.5LT, Sigma, St. Louis, MO), Potassium choride (KCl) (P9541-500G, Sigma, St. Louis, MO), Poly Vinylidene Difluoride Membrane (PVDF membrane) (P2938, Sigma, St. Louis, MO), Na2HPO4 (S3264-500G, Sigma, St. Louis, MO), Non-fat milk (Marvel, Tesco, Ireland), Sodium dodecyl sulfate (SDS) (L4390-25G, Sigma, St. Louis, MO), PBS (20012-019, Gibco, Carlsbad, CA), Sodium chloride (NaCl) (S3014-500G, Sigma, St. Louis, MO), Sucrose (84097-500G, Fluka, St. Louis, MO), Tetramethylethylenediamine (TEMED) (T7024-25ML, Sigma, St. Louis, MO), Triton X-100 (P9284-500ML, Sigma, St. Louis, MO), Tris (0497-1KG, Amresco, Cochran Solon, OH), Tris-EDTA (TE) (T9285-100ml, Sigma, St. Louis, MO), Tween-20 (P7949-500ML, Sigma, St. Louis, MO), Whatman papers (grade 5, 1003-919, Whatman, GE, UK), Whatman papers (grade 3, 1003-917, Whatman, GE, UK), Isopropanol (I9516-500ml, Sigma, St. Louis, MO), Acetic Acid (695092, Sigma) Acrylamide 30% Bisacrylamide solution 37.5 (161-0158-500ml, Bio-Rad) Glycine (G080060-1kg, Fisher) Hydrochlorlic acid (258148-500ml, Sigma) Immobilon P polyvinylidene difluoride membrane (PVDF) (P2938, Sigma) Potassium chloride (P9541-500g, Sigma) Potassium phosphate dibasic trihydrate (P9666-100g, Sigma) Precision Plus Protein Dual Colour Standards (161-0374-0.5ml, Bio-Rad) Sodium hydroxide (S8045-500g, Sigma) Sodium phosphate dibasic (S5136-500g, Sigma) Tris Base (BP152-1kg, Fisher) Triton X-100 (T9284-500ml, Sigma) Tween 20 (P7949-500ml, Sigma) 0.25% Trypsin/EDTA solution (T4049-100ml, Sigma) 0.4% Trypan blue solution (T8154-100ml, Sigma) B27 (17504-044-10ml, Invitrogen) D-(+)-glucose (G8270-1kg, Sigma) Dimethylsulphoxide (DMSO) (D8418-250ml, Sigma) DMEM-F12 (SH3000404, Fisher) Dulbecco's PBS (14190-094-500ml, Invitrogen) Fetal Bovine Serum (FBS) heat inactivated (S1900-500ml) Filter paper grade 3 (1003-917, Whatman) Formaldehyde 37% solution (5339988500ml, Sigma) Hyclone Hypure cell culture grade water (HYC-001-216G-500ml, Thermoscientific) Laemmli (sample) buffer (161-0737-30ml, Bio-Rad) L-glutamine (G7513-100ml, Gibco) Neurobasal A medium

(10888-022-500ml, Invitrogen) Optimem reduced serum medium (11058-021-500ml, Invitrogen) Penicillin/streptomycin (P4333-100ml, Sigma) Phosphate buffered saline (PBS) (20012-500ml Invitrogen) Poly-L-lysine hydrobromide (P1399-25mg, Sigma), Dantrolene Sodium Salt (D9175-100MG, Sigma). U0126 (V1121, Promega), MK2206 (S1078-5mg, Selleckchem)

1.2 Labware

0.2 µm single-use filter units (16534, Sartorius Stedim), 35 mm Petri dishes (1171734, Lennox), Borosilicate coverglass 13 mm diameter (631-0149, VWR), Disposable and sterile cell scrapers (08-100-241, Fisher), Microscope slides 76 mm x 26 mm (MNJ-150-030U, Fisher), Nunc MaxiSorp® flat-bottom 96 well plate (DIS-971-090X, Fisher), Nunc 24-well plates (TKT-190-110Y, Fisher), Nunc 6-well plates (TKT-190-110E, Fisher), single use filter units Millex GP (SLGP033RS, Millipore), T-150 cm^2 cell culture flasks (430825, Corning), T-25 cm^2 cell culture flasks (430639, Corning), T-75 cm^2 cell culture flasks (430641, Corning), Millicell Cell Culture Insert 30mm hydrophilic PTFE 0.4µm (PICM0RG50, Millipore)

1.3 Electronic Equipment

Fujifilm LAS-3000 Intelligent Dark-box, E24 Incubator Shaker (New Brunswick Scientific E24 Incubator, Mason Technology), Small bench centrifuge (Hermle, Mason Technology), Sonicator (Sonics, Vibracell), Water bath (UAB 12 EU, Grant) Epoch Microplate Spectrophotometer (BioTek, Mason Technology).

1.4 Software and statistical tests

Immunoflourescent staining of cerebellar slices was quantified using EBImage (http://www.bioconductor.org/help/biocviews/release/bioc/html/EBImage.html), a software package run through the 'R' statistical graphing environment. Western blots and migration assays were analysed using ImageJ (http://rsbweb.nih.gov/ij/download.html) while flow cytometry data was analysed using FlowJo (Tree Star Inc., http://www.flowjo.com/download-flowjo/). All graphs and statistical analyses were generated through GraphPad Prism. Bartlett's test for equal variances was used to establish normality and differences between mean sample values were examined using ANOVA followed by Newman-Keul's post-hoc test unless otherwise stated.

Primary Antibodies				
Antibody	**Host**	**Manufacturer**	**Dilution**	**Purpose**
anti-pERK	Rabbit	Millipore	1/3000	Western blotting
anti-ERK	Mouse	Millipore	1/3000	Western blotting
anti-pAkt	Rabbit	Cell Signalling	1/3000	Western blotting
anti-GFAP	Rabbit	Dako	1/5000	Western blotting
anti-GFAP	Mouse	Millipore	1/5000	Western blotting
anti-Actin	Mouse	ECM Biosciences	1/1000	Western blotting
anti-MOG	Mouse	Millipore	1/3000	Western blotting
anti-MBP	Rabbit	Abcam	1/500	Immunohistochemistry
anti-NFH	Chicken	Millipore	1/1000	Immunohistochemistry
anti-CD3	Rat	eBioscience	to 1µg/ml	Cell Stimulation
anti-CD28	Rat	eBioscience	to 1µg/ml	Cell Stimulation
anti-CD4 Pc-Cy7	Mouse	eBioscience	1/100	Flow Cytometry
anti-CD3 PE conjugated	Mouse	eBioscience	1/100	Flow Cytometry
anti-CD25 FITC	Rat	BD Biosciences	1/50	Flow Cytometry
anti-Foxp3 APC	Mouse	eBioscience	1/100	Flow Cytometry
Secondary Antibodies				
Antibody	**Host species**	**Manufacturer**	**Dilution**	**Purpose**
anti-Mouse Dylight 488	donkey	Jackson ImmunoReseach	1/1000	Immunohistochemistry
anti-Rabbit Dylight 488	donkey	Jackson ImmunoReseach	1/1000	Immunohistochemistry
anti-Rabbit DyLight 549	donkey	Jackson ImmunoReseach	1/1000	Immunohistochemistry
anti-Mouse Alexa 633	goat	Invitrogen	1/1000	Immunohistochemistry
anti-Rabbit Alexa 633	goat	Invitrogen	1/1000	Immunohistochemistry
anti-Mouse Dylight 549	goat	Jackson ImmunoReseach	1/1000	Immunohistochemistry
anti-Mouse HRP	Goat	Sigma	1/10000	Western blotting
anti-Rabbit HRP	Donkey	GE Healthcare	1/10000	Western blotting

Table 2.1 Primary and secondary antibodies used

Treatments Used				
Compound	**Solvent**	**Supplier**	**Stock Concentration**	**Purpose**
FTY720	DMSO	Novartis, Basel	10mM	In vivo use
pFTY720	DMSO	Novartis, Basel	10mM	In vitro use
S1P	DMSO	Novartis, Basel	10mM	In vitro use
AUY954	DMSO	Novartis, Basel	10mM	In vitro use
MNP301-304	$d.H_20$	Genscript	14mg/ml	In vitro use
MK2206	DMSO	Selleck Chemicals	10mM	In vitro use
U0126	DMSO	Promega	10mM	In vitro use
Dantrolene	DMSO	Sigma	10mM	In vitro use
EGTA	$d.H_20$ (+NaOH to pH 8.0)	Sigma	0.5M	In vitro use

Table 2.2 Treatments used

2. *In vivo* Methods

2.1 *In vivo* MOG-immunisation of C57BL/6 mice

C57BL/6 mice (Harlan UK) were maintained in the BioResources unit at Trinity College, Dublin, Ireland under veterinary supervision throughout the study. Experiments were performed under a license issued by the Department of Health (Ireland) and in accordance with the guidelines laid down by Trinity College Dublin ethical committee. Female C57BL/6 mice (8-10 week old) were sub-cutaneously injected at the base of the spine with 200µl of myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}, AnaSpec) at a concentration of 500µg/ml in Complete Freund's Adjuvant (Chondrex). Mice were also administered an interperitoneal injection of 200µl pertussis toxin (PTX) at 2.5µg/ml on day 0 and again on day 2. An interperitoneal injection of FTY720 was given at 8µg/day for 10 days (days 0-9). Spleens were taken on day 10 after immunisation.

3. Cell Culture

3.1 *In vitro* culture and stimulation of MOG reactive T cells.

MOG-immunised C57BL/6 mice were sacrificed by cervical dislocation and an incision was made on the left flank between the hip and rib-cage. Spleens were then removed in order to prepare a single cell suspension. Spleens from 2D2 transgenic mice (Bettelli et al. 2003) were kindly provided by Dr. Denise Fitzgerald, Queen's University, Belfast. These 2D2 spleens were extracted in Dr. Fitzgerald's laboratory in Belfast and immediately transported to Dublin on ice and in tubes contained ice-cold RPMI media. A single cell suspension was created from isolated spleens by crushing the splenic capsule, in 5ml PBS, with the rough end of a syringe plunger and agitating until the majority of cells floated free. Cell suspension was passed through a 40µm mesh filter and into a 50ml tube. Tubes were centrifuged at approximately 1300RPM for 5 minutes to form a cell pellet. The PBS was decanted and the cell pellet was resuspended in 5ml ACK buffer (0.15M NH_4Cl, 10mM $KHCO_3$ and 1mM EDTA) for maximum 5 mins to lyse erythrocytes. ACK was quenched in 25ml RPMI-1640 media (Gibco) supplemented with 10% FCS (Biosera), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma) and 0.1% β-mercaptoethanol (Sigma) (dubbed R-10 media). Samples were centrifuged as previous and resuspended in R-10 at desired concentration. MOG reactive lymphocytes from MOG-immunised mice or 2D2 transgenic animals ($2x10^6$cells/ml) were incubated with 25µg/ml MOG_{35-55} peptide for 48 hours with or without pFTY720 (1nM-1µM). Cells from control mice ($2x10^6$cells/ml) were stimulated with plate-bound mouse anti-CD3 and anti-CD28 (BD Biosciences) at

1µg/ml for 48 hours. For ELISA and FACS studies, cells were activated as above with or without pFTY720 (1nM-1µM), TGF-β (5ng/ml) and/or IL-2 (20ng/ml) for four days at 37°C and 5% CO_2 before removal of media for ELISA and staining of cells for FACS analysis.

3.2 Organotypic Cerebellar Slice Culture

Experiments were conducted using tissue isolated from postnatal day 10 (P10) C57BL/6 mice in accordance with EU guidelines and protocols approved by the Trinity College Dublin ethics committee. The cerebellar slice culture was based on published protocols (Birgbauer et al., 2004). Briefly, the cerebellum and attached hind-brain tissue were removed from the skull and placed directly into ice cold Opti-mem media. After 2-3 minutes the cerebellum was carefully removed from neighbouring tissue and placed on mount in preparation for chopping. 400 µm parasagittal slices of cerebellum were cut using a McIlwain tissue chopper. Slices were viewed under a dissection microscope and separated as necessary using small gauge needles. All separated tissue was removed to the laminar flow hood and then 5-6 slices were transferred to each cell culture insert (Millicell PICMORG50). Slices were cultured using an interface method with 1 mL of medium per 35 mm well. For the first 3 days *in vitro* (DIV), slices were grown in serum-based medium (50% Opti-Mem, 25% Hanks' buffered salt solution (HBSS), 25% heat-inactivated horse serum and supplemented with 2 mM Glutamax, 28 mM D-glucose, 100 U/mL penicillin/streptomycin and 25 mM HEPES) at 35.5C and 5% CO2. After 3 DIV, slices were transferred to serum-free medium (98% Neurobasal-A and 2% B-27 (Invitrogen), supplemented with 2 mM Glutamax, 28 mM D-glucose, 100 U/mL penicillin/streptomycin and 25 mM HEPES). To induce demyelination, 12 DIV cultures were transferred to fresh serum-free medium ready for treatment

3.3 Co-culture of Lymphocytes and Cerebellar Slices

Organotypic cerebellar slices were cultured according to the method reported previously. Cultured spleen cells were counted and washed by centrifugation before being resuspended at a concentration of $1x10^6$cells/ml in organo-serum media. The cells were washed again by centrifugation and the resuspended lymphocyte cell suspension (5µl, ~$5x10^4$cells) was added directly onto each slice and co-cultured for 48 hours at 35.5°C and 5% CO_2. As a positive control, slices were treated with 0.35mg/ml lysolecithin (LPC: Sigma, L-4129) for 18 hours at 35.5°C and 5% CO_2

before being moved to fresh media for the remainder of the 48hr treatment (**Figure 2.1**).

3.4 Astrocyte culture

Primary cortical astrocyte cultures were prepared using post-natal day one Wistar rats of either sex (Bioresources Unit, Trinity College Dublin), in accordance with the Animals Act 1986 (Scientific Procedures) Schedule I guidelines. Briefly, the brain was freed of meninges and cortices were dissected in warmed DMEM/F12 (Biosera, East Sussex, UK), supplemented with 10% heat inactivated FBS (Biosera) and 1% penicillin/ streptomycin (100 µL·mL−1; Invitrogen) (sDMEM). Tissue was triturated and passed through a sterile nylon mesh cell strainer (40 µm; BD Biosciences). Cell suspension was centrifuged and the pellet resuspended in sDMEM/F12. Cells were plated on poly-L-lysine (40 µg·mL−1 in sterile H2 O: Sigma Aldrich, Germany) coated T75 culture flasks (Cat #83.1813.002, Sarstedt AG, Nümbrecht, Germany). When confluent, the flasks were shaken at 200 rpm for 3 hours at 37°C in an orbital shaker (Excella E24, New Brunswick Scientific, Boulevard Enfield, CT, USA) and non-astrocyte cells removed. The astrocyte layer was incubated with 0.1% trypsin-EDTA in serum free DMEM/F12 for 5-20 minutes at 37°C until astrocyte layer lifted and sDMEM/F12 was added to the flasks to inhibit the trypsin. The cell suspension was collected, centrifuged and resuspended in sDMEM/F12 and plated in 6 well plates or on migration assay coverslips. Cells were grown until confluent and were starved in serum-free media for 3 hours prior to treatment. Cells were pre-treated with antagonists/inhibitors (BYR185, Novartis Pharma; MNP301, Genscript; U0126, MK2206, Selleckchem; EGTA, Sigma; Dantrolene, Sigma.) for 1 hour at concentrations indicated in figure legends. Agonists (AUY954, pFTY720 and S1P, Novartis Pharma.) were added after for times and at concentrations indicated in figure legends.

4. Cellular and Biochemical assays

4.1 Immunofluorescent Staining

After incubation all slices were washed in phosphate buffered saline (PBS: Sigma). 1ml of PBS was added to the wells of a 6 well plate and the inserts transferred to this plate. 1ml of PBS was then added on top of the insert also (1ml above and below). This wash lasted 2 minutes. Slices were then fixed in 4% paraformaldehyde in PBS, 1ml above and below, at 4°C for 10 minutes. They were washed again in PBS at 4°C for 5 minutes before being blocked and permeabilized in a solution of 0.25% Triton X

(Sigma) and 10% BSA in PBS, 1ml above and below, for 48-72 hours at 4°C. The blocking and permeabilization buffer was removed and the all the slices on the insert were cut out, retaining them on the underlying membrane. They were then stained with 50µl each (300µl for six slices on an insert) of staining cocktail consisting of anti-neurofilament H (1/1000 dilution: Millipore, MAB5539) and anti-myelin basic protein (1/200 dilution: Abcam, ab40390) and incubated overnight at 4°C (**Table 2.1**). Slices were washed in PBS and 0.1% BSA for 2x 30 minutes and then stained with 50µl each of a DyLight 549 conjugated anti-chicken secondary antibody (1/1000: Jackson Immunoresearch) and DyLight 488 anti-rabbit secondary antibody (1/1000: Jackson Immunoresearch) cocktail (**Table 2.1**). These were incubated overnight in the dark at 4°C before being washed 2x 30mins in PBS in the dark. Slices were placed on microscope slides and mounted with coverslips using OCT (Optimal Cutting Temperature compound). Slides were stored in the dark at room temperature before being imaged.

4.2 Confocal Microscopy and Fluorescent Imaging

Confocal images captured for the quantitative measurement of immunofluorescent staining were 12 bit .tiff files of 1024 x 1024 pixel resolution. They were captured using a 40X/1.30 oil-immersion lens or a 20X/1.30 lens (Zeiss) on a LSM 510 Meta microscope. Three to six images were captured per slice. Image analysis was conducted using the software package EBImage (http://www.bioconductor.org/help/biocviews/release/bioc/html/EBImage.html) run through the statistical programming environment, R. The software package allowed analysis of red, green and blue channels separately. Each of the 1024 x 1024 pixels within the images was assigned an intensity value between 0 and 1. Neurofilament H staining (red) labels axonal processes and was used as a marker in order to identify axons so that MBP staining separate from axons could be distinguished and discounted. This MBP staining, co-localized with neurofilament H, represents neuronal MBP staining (NeuroMyelin). The fluorescence intensity thresholds were set to remove background staining so that only those pixels representing specific staining are included in the analysis measurements. For MBP, the percentage of green pixels which were co-localized with red (neurofilament H) was calculated. This percentage was then multiplied by the average green pixel fluorescence intensity to give the NeuroMyelin Score. In experiments where co-localization was not possible to compute, due to excessive debris etc., the total myelin fluorescence in each image was calculated. In these instances the number of green pixels over threshold as a percentage of the total number of pixels in the image was calculated. This percentage

was multiplied by the average fluorescence intensity (between 0 and 1) of all green pixels to give a total myelin score.

4.3 Flow Cytometry

For flow cytometry, cultured spleen cells were washed in PBS supplemented with 1% FCS, 1% Sodium Azide and 3mM EDTA (FACS buffer) and stained with a cocktail of anti-CD3 PE, anti-CD4 APC-Cy7 and anti-CD25 FITC (eBioscience). Cells were then fixed and permeabilized using eBioscience FoxP3 staining kit (77-5775-40) and stained with anti-Foxp3 APC (**Table 2.1**). Samples were examined using a BD LSRFortessa flow cytometer and analyzed using FlowJo software (Treestar).

4.4 ELISA

Supernatants from cell culture were removed and examined for their cytokine content using ELISA kits for IL10 (DY417), IFNγ (DY485), IL6 (DY406) and TNFα (DY410) according to the manufacturer's instructions (R&D systems, http://www.rndsystems.com). Briefly, the capture antibody was diluted to the indicated working concentration and 100µl was added to the wells of a 96 well plate (Nunc MaxiSorp®, DIS-971-090X, Fisher) and allowed to bind to the plate overnight at room temperature. The contents of the wells were removed and washed 3 times by flooding with PBS containing 0.05% Tween 20® followed by complete removal of wash buffer. The plate was then blocked by adding 300µl of 1% BSA solution in PBS to each well and incubating for >1 hour.. Standards were diluted in PBS or Tris-buffered saline (TBS) with 0.1-1% BSA depending on the kit. A seven-fold serial dilution of standards was performed using the reagent diluent. Wells were washed as above and 100µl of samples and standards were added to appropriate wells. After a >2 hour incubation at room temperature with gentle agitation on an orbital shaker wells were once again washed 3 times. Capture antibody was diluted in reagent diluent as described in manufacturer's instruction and 100µl was added to each well. After a further >2 hour incubation with agitation, wells were washed x3 and Streptavidin-HRP (100µl at working dilution suggested on vial) was added to each well. The plate was kept out of the light and incubated at room temperature for a further 20 minutes and then again washed x3. 100µl of TMB substrate solution (1:1 ratio of H_2O_2 and tetramethylbenzidine) was added to each well followed by an additional incubation in the dark. The development of the colour was monitored until it was deemed that the upper standards were approaching saturation whereupon 50µl of 2N H_2SO_4 was added to stop the reaction. The optical density (O.D.) of the contents of the plate was read using an EPOCH microplate spectrophotometer (BioTek, Mason Technology) and

GEN 5 software. The plate was read at 450nM and 570nM. Subtracting the values seen with 570nM from those seen at 450nM allows for corrections in optical variations in the plate. The cytokine content of each sample was quantified by comparing the O.D. of these samples to the values of the known standards when plotted on a standard curve.

4.5 Migration Assay

Coverslips of 13mm diameter (VWR) were poly-L-lysine coated. Approx. 0.5mm square pieces of silicon were cut and placed on one surface of the coverslip and they were then placed into individual 35mm diameter petri dishes (BD biosciences). The squares of silicon increased the adherence of the coverslip to the base of the dish. Strips of silicon were then cut into 0.5mm widths using a McIlwain tissue-chopper and one strip was laid down through the centre of each coverslip. 2 x 10^5 cells suspended in 200µl sDMEM/F12 were pipetted each side of the silicon strip and allowed to adhere for approx. 1 hour before flooding the dish with 2ml of sDMEM/F12. Cells were allowed to grow until confluent (1-2 days) before treatment. Astrocytes for migration assays were serum-starved for 3 hours prior to all treatments. Cells were pre-treated with antagonists/inhibitors (BYR185, Novartis Pharma; MNP301, Genscript; U0126, MK2206, Selleckchem; EGTA, Sigma; Dantrolene, Sigma.) for 1 hour at concentrations indicated in figure legends. Agonists (AUY954, pFTY720 and S1P, Novartis Pharma.) were added after 1 hour incubation of inhibitor for times indicated in figure legends. Immediately after treatment of astrocytes with agonists the silicon strips were removed from the coverslips. 10 to 12 images from each coverslip were captured at 4x magnification as a basal control. After 24 hours of incubation at 37° and 5% CO_2, 10-12 images were again captured. The difference between the width of the cell-free strip at 0 and 24 hours was measured manually using ImageJ. Briefly, a rectangular area was drawn in the cell-free area of the strip and the width of this area was taken as the distance between the cell borders on each side. For images with irregular borders, the average width of numerous smaller areas was used as the measure. All measurements were plotted as a percentage of untreated control movement (**Figure 2.2**).

4.6 SDS-PAGE and Western Blot

Cerebellar slices were scraped from the culture membrane and suspended in ice-cold PTxE buffer (PBS, 1% Triton-x, 1mM EDTA) using mechanical homogenisation and sonication. Astrocyte cell samples were rinsed in ice-cold PBS and scraped in ice-cold

PTxE buffer and homogenised. BCA assays (https://www.piercenet.com/instructions/2161296.pdf) were performed to estimate total protein concentration in each sample. If necessary concentrations were normalised by adding additional PTxE buffer so that all samples contained equivalent protein levels. Tissue samples were mixed 1:1 in sample buffer (1:20 β-mercaptoethanol in Laemmli buffer (Bio-Rad, 161-0737) and denatured at 95°C for 5mins. Samples were loaded into a 10% poly-acrylamide gel and the protein content of the gel was transferred to an Immobilon-P PVDF membrane (Millipore, IPVH00010) for 90mins at 50mA. The membrane was blocked in blocking buffer (5% milk in PBS-T: PBS, 0.1%Triton-x) for 1hr at room temperature or overnight at 4°C. Primary antibodies against MOG (Millipore, MAB5680), MBP (Abcam ab40390) phospho-ERK, total ERK, phospho-Akt (Cell Signalling), GFAP (Millipore) or Actin (ECM Biosciences, AM2021) were diluted in blocking buffer and incubated for 1hr at room temperature or overnight at 4°C. Blots were given 3x5min washes in PBS-T before HRP conjugated secondary antibodies against mouse (Sigma, A8924) or rabbit (GE Healthcare, NA934) were diluted 1/5000 in blocking buffer and added to blots. They were then incubated for up to 2hrs at room temperature. At least 3x5min washes were performed before blots were developed using Immobilon HRP chemiluminescent substrate (Millipore, P36599A). Digital images of blots were taken using a Fujifilm LAS-3000 Intelligent Dark-box. Following imaging, blots that were to be re-probed for loading controls were stripped using. These images were analysed by densitometry using ImageJ software. Each lane was outlined and the intensity of the band was measured, background signal was removed and the value for each band was normalised to its own loading control value.

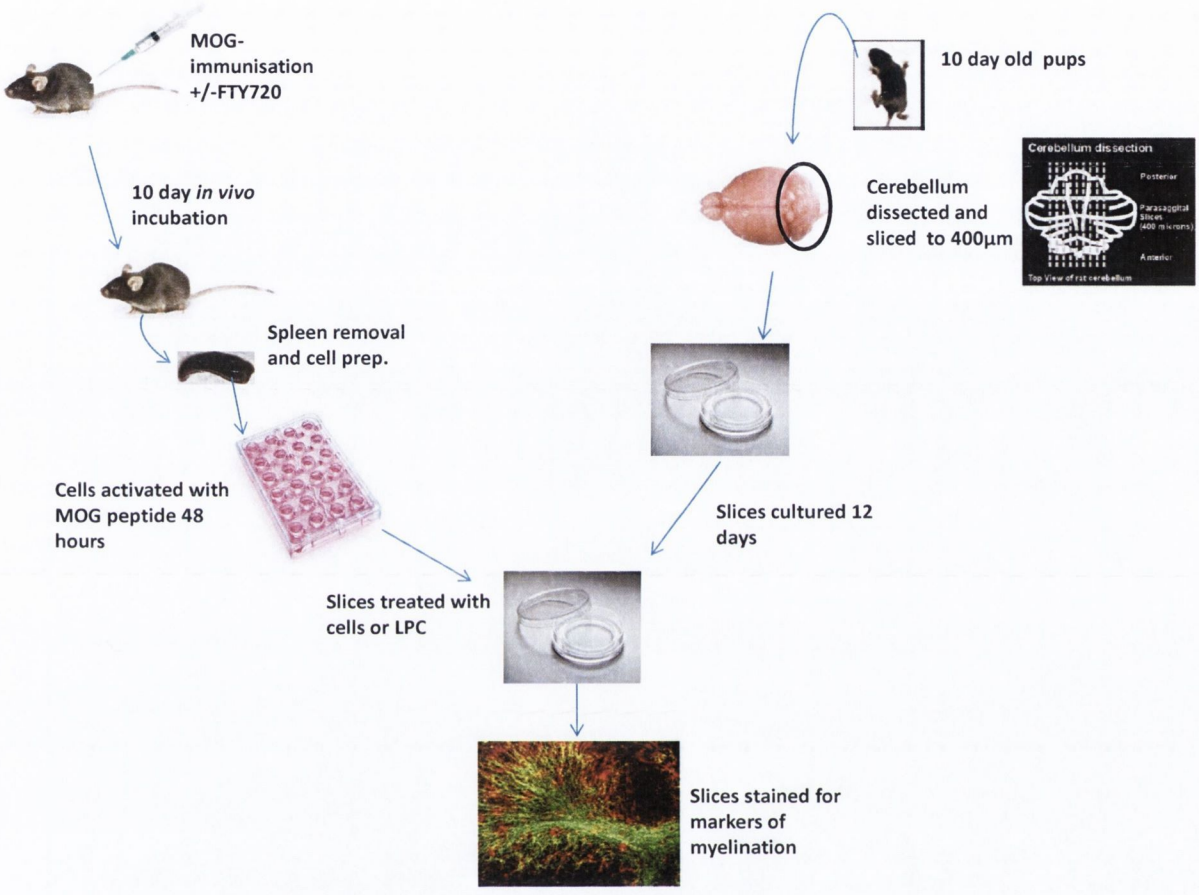

Figure 2.1 Cerebellar slice and splenocyte co-culture schematic.

Spleen cells from MOG-immunised mice are cultured in the presence or absence of pFTY720. Cerebellar slices are cultured from P10 pups before the addition of splenocytes to slices. After 48hours of co-culture slices are fixed and stained for MBP.

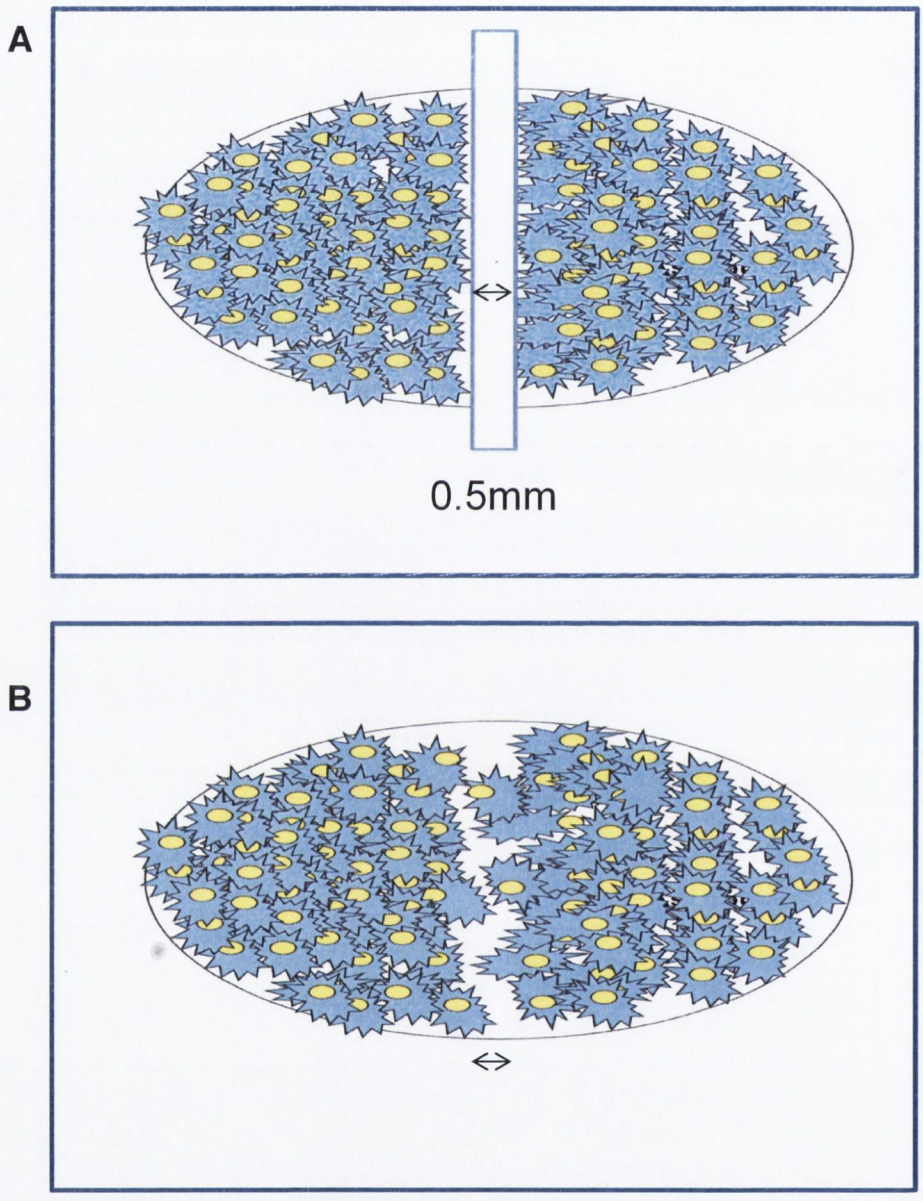

Figure 2.2 Astrocyte migration assay

Astrocytes are grown on a coverslip with a silicon strip 0.5mm in width down the centre. Once the strip is removed the astrocytes migrate into the vacant space. A measurement of the width of the vacant space is taken at 4h and 24h post-treatment. The difference in the two values represents the distance migrated

Chapter 3. The impact of S1PR1 modulation on the phosphorylation of ERK in rat astrocytes

Chapter Aims:

- To determine the S1PRs responsible for the activation of ERK by S1P and pFTY720 in astrocytes.

- To examine the effects of MNP301 treatment on S1PR1-induced ERK phosphorylation.

- To demonstrate the specificity of MNP301 modulation of S1PR1-induced pERK

- To preclude the possible contribution of calcium signalling to S1PR1-associated ERK activation

- To isolate the active site within the MNP301 amino-acid sequence.

Abstract

ERK phosphorylation has been identified as a signalling event that is central to the survival and proliferation of numerous cell types. S1PR activation induces potent ERK phosphorylation in astrocytes. This phosphorylation is thought to be mediated through S1PR1; however, there is evidence to suggest that S1PR3 activation may also induce activation of this signalling cascade. In this chapter, it is demonstrated that S1PR3 likely induces significant phosphorylation of ERK in astrocytes. This is due to an inability of BYR185, an S1PR1-specific antagonist, to attenuate ERK activation induced by the pan-S1PR agonists S1P and pFTY720, whereas, the antagonist BYR185 attenuates pERK induced by the specific S1PR1 agonist AUY954. Previous work in this lab has shown that a pathway specific modulator of S1PR1 signalling, MNP301, can reduce calcium signalling in astrocytes while leaving cAMP signals unaffected. This chapter describes that this MNP301 peptide can also inhibit the phosphorylation of ERK induced by AUY954 activation of S1PR1. Furthermore, it is shown that these effects are mediated by the direct and specific activity of MNP301 on S1PR1-activated pERK and not by general inhibition of ERK signalling or by reduction in calcium release. Finally, the amino-acid sequence necessary for activity of the MNP301 peptide was examined. Taken together, these data support the hypothesis that MNP301 is a selective inhibitor of intracellular signals propagated by S1PR1.

1 Introduction.

1.1 ERK Signalling

The mitogen-activated protein kinase (MAPK) signalling pathways are involved in multiple signalling cascades that regulate numerous cellular functions. Three of the best described MAPK pathways are the p38, c-Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways. The basic components of most MAPK pathways involve the activation via phosphorylation of a MAPK kinase kinase (MAP3K) by a G-protein or other stimulus (Kolch 2000). This in turn induces the phosphorylation of a MAPK kinase (MAP2K) bringing about the subsequent phosphorylation of the MAPK. These MAPKs (e.g. p38, JNK, ERK etc.) can then regulate transcription factors or the activation of a variety of cytoplasmic substrates (Kolch 2000).

The ERK family of MAP kinases consists of numerous members, the best described of which are ERK1 (MAPK3 or p44) and ERK2 (MAPK1 or p42). This ERK1/2 pathway is considered the classical mammalian MAPK cascade. ERK1/2 were originally identified as protein kinases with the ability to phosphorylate microtubule-associated protein-2 and myelin basic protein (Boulton & Cobb 1991). These kinases were found to be expressed ubiquitously in rat tissue and in especially high concentrations in the brain and spinal cord. Both kinases were shown to be conserved across many species, including human, mouse, dog, chicken and frog (Boulton & Cobb 1991). Activation of the ERK pathway is achieved through binding of a ligand to a cell-surface receptor (**Figure 3.1**), be it receptor tyrosine kinase (RTK) or G protein-coupled (GPCR) (Campbell et al. 1998). Son of sevenless (SOS) is a Ras-activating guanine nucleotide exchange factor that binds Ras at the plasma membrane and stimulates the switch from a Ras-GDP to a Ras-GTP bound state (Geyer & Wittinghofer 1997) (**Figure 3.1**). In this active form Ras can interact with a range of effectors such as the members of the Raf family, B-Raf, Raf-1 and A-Raf. The activation of Raf by Ras is a complex process that appears to differ from family member to family member.

Raf-1 is the most studied of the Raf proteins but its method of activation is not yet fully understood. Raf-1 binds to Ras with high-affinity but, in order to enhance Raf-1's catalytic activity, binding must occur at the plasma membrane (Kikuchi & Williams 1994). Two domains on the Raf-1 N-terminus allow Ras binding, the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) (Brtva et al. 1995). RBD interaction allows for translocation of the protein complex to the cell membrane however the CRD

needs to be recruited in order to bring about Raf-1 activation (Hu et al. 1995; Roy et al. 1997). In addition, Raf-1 also requires phosphorylation at two specific residues in order to achieve full activation. Ras signals activate phosphoinositide 3-kinase (PI3K), products of which activate Rac and in turn p21cdc42/rac1-activated serine/threonine kinase (PAK). This induces the phosphorylation of Raf-1 at serine-338 (King 1998). Src family kinases can cause phosphorylation at tyrosine-341 (Mason et al. 1999). Phosphorylation of serine-259 by Akt, a protein also activated by PI3K, may also supply an inhibitory signal to Raf-1 activation (Zimmermann & Moelling 1999). The presence of numerous phosphopeptides upon Raf-1 activation indicate that other, as yet un-described phosphorylation events may occur in order to achieve full Raf-1 activation. In comparison to Raf-1, the activation of B-Raf is relatively simple. It appears that binding to Ras-GTP alone is sufficient to bring about B-Raf activation (Mason et al. 1999). Site-specific phosphorylation is not necessary as B-Raf contains an aspartate residue in the place of Raf-1's tyrosine-341 and constitutive phosphorylation at the equivalent to serine-338 (Mason et al. 1999). A-Raf activation has been little explored but appears to be somewhat similar to Raf-1 insofar as both a Ras signal and phosphorylation at a residue corresponding to tyrosine-341 are required to achieve full activation (Marais et al. 1997; Mason et al. 1999).

Activated Raf can bind to a downstream kinase known as MAPK/ERK kinase (MEK) which acts as the MAP2K in the ERK pathway (**Figure 3.1**). MEK is found in two functionally indistinguishable forms, namely MEK1 and MEK2 (Seger & Krebs 1995). All Raf isoforms can activate either form of MEK and MEK1/2 can both activate the same downstream kinases within the pathway. MEK1/2 are dual-specificity kinases, insofar as they can phosphorylate both threonine and tyrosine residues (De Luca et al. 2012). Within this signalling cascade, they have the ability to phosphorylate ERKs within a Thr-Glu-Tyr (TEY) motif in their activation loop, thereby activating this downstream kinase (De Luca et al. 2012) (**Figure 3.1**).

1.2 ERK isoforms

ERK itself comes in two different isoforms, namely ERK1 (also known as p44 or MAPK1) and ERK2 (p42 or MAPK2) (Boulton & Cobb 1991; Boulton et al. 1991), of 42 and 44 kDa respectively. These proteins display 90% sequence homology (Boulton et al. 1991) and were long considered to perform similar functions within the mammalian system but this view has shifted and experiments indicate that the two proteins have distinct functions (Lloyd 2006). Knockout experiments give some insight into the differing function of the ERKs. ERK2 -/- die early in development whereas ERK1 -/- are

viable and only display mild developmental abnormalities, however, there is no detectable upregulation of ERK2 in these animals (Lloyd 2006). Despite this, there is evidence that ERK2 activation is increased in an ERK1-/- and this can lead to improved long-term potentiation and learning (Mazzucchelli et al. 2002). It is thought that ERK1 may inhibit the activity of ERK2, (Vantaggiato et al. 2006) although the exact mechanism by which this occurs is not clear. Fibroblasts from ERK1-/- animals display an increased rate of proliferation *in vitro,* likely due to increased ERK2 activation, while cells from ERK2-/- animals proliferate poorly, again implying that ERK1 acts as an inhibitor of ERK2 activation (Vantaggiato et al. 2006). In its resting state ERK is directly bound to MEK and through this interaction is tethered in the cytoplasm. Upon activation, the phosphorylation of tyrosine residues on ERK causes its dissociation from the complex and allows for translocation to the nucleus. The movement of ERK from cytoplasm to nucleus occurs via three different mechanisms. Passive diffusion allows an ERK protein monomer to pass into the nucleus (Adachi et al. 1999) while homodimers of the phosphorylated and phosphorylated or unphosphorylated partners (Khokhlatchev et al. 1998) are actively transported to the nucleus via a Ran-dependent mechanism (Adachi et al. 1999). A third mechanism of transport involves interaction of pERK with a nuclear pore complex through which it gains entry to the nucleus (Matsubayashi et al. 2001).

1.3 Downstream substrates of ERK

Once the pERK protein has passed into the nucleus it is able to activate downstream signalling molecules that in turn activate a series of transcription factors. These intermediary signalling proteins activated within the nucleus are called mitogen and stress activated protein kinases (MSK) (Hauge & Frödin 2006) (**Figure 3.1**). pERK induces the phosphorylation of these kinases and they in turn activate transcription factors such as CREB, ATF1, Histone H3 and HMG-14 through the phosphorylation of serine residues (Hauge & Frödin 2006). Ribosomal S6 kinase (RSK) is phosphorylated by pERK outside the nucleus but is then translocated across the nuclear membrane whereupon it can phosphorylate serine residues on Jun-Fos, SRF and CREB also activating these transcription factors (Hauge & Frödin 2006). pERK directly activates the transcription factors ELK-1 and Myc-Max as well as the translational inhibitors BRF-1 and UBF. In the extra-nuclear region it can also phosphorylate MNK which is an activator of eLF4E, another inhibitor of translation (Hauge & Frödin 2006).

1.4 Enhancers, adaptors and inhibitors of the ERK pathway

The activation of numerous downstream substrates is dependent on ERK signalling yet these substrates perform a variety of functions. Scaffolding and inhibiting proteins interact at different points in the pathway, augmenting or attenuating the pathway activation. These proteins include the scaffolding proteins; kinase suppressor of Ras-1 (KSR1), connector–enhancer of KSR (CNK) and MEK partner 1 (MP1), and the inhibiting protein; Ras-kinase inhibiting protein (RKIP) (**Figure 3.1**).

1.4.1 KSR1

KSR1 was first identified in a genetic screen of *Drosophila melanogaster* as a suppressor of activated Ras protein (Morrison 2001) and it has been found to share much sequence homology with Raf-1 but lacks the catalytic domains that are deemed to be necessary for enzymatic activity. In addition, the bulk of evidence supports the theory that KSR1 functions as a scaffold within the Raf/MEK/ERK pathway. It is thought that MEK may be constitutively bound to KSR but that Raf and ERK bind in an activation dependent manner (Morrison 2001). Low levels of KSR expression augment ERK signalling whereas over-expression disrupts this pathway, a feature of many scaffolding proteins (Cacace et al. 1999). ERK signalling is usually a short-term event but KSR may function to promote enhanced and sustained signaliing of the pathway, resulting in a different biological outcome (Kolch 2005).

1.4.2 CNK

Connector-enhancer of KSR (CNK) was also first identified in *Drosophila* and was found to enhance the activity of dominant-negative form of KSR by inhibiting Ras but not Raf activation (Therrien et al. 1998). While CNK has no catalytic domains, it does have numerous protein-protein interacting domains, indicating it functions as an adapter-protein within the ERK pathway (Therrien et al. 1998). Further investigations into Ras signalling in the *Drosophila* system showed that CNK can regulate the ERK pathway via a Raf interacting domain on its C-terminus in addition to a non-ERK pathway via its N-terminus (Kolch 2005). This duality of function could allow for precise modulation of signalling events downstream of Ras. However, no direct interaction between mammalian Raf-1 and the human homologue of *Drosophila* CNK has been found meaning the exact mechanism of action in mammals is as yet unclear (Kolch 2000).

1.4.3 MP1

MEK partner 1 (MP1) is another adapter protein that has been found to function in the Raf/MEK/ERK pathway. It preferentially interacts with MEK1 and ERK1 and localises ERK1 signalling to endosomes (Schaeffer et al. 1998). Promoting ERK1 activation in this manner may promote the inhibition of ERK2 and dampen the proliferative signal of the ERK/MAPK pathway. MP1 may form part of a larger scaffolding complex with other proteins such as MAPK organizer-1 (MORG-1) (Kolch 2005). MORG-1 stably binds to MEK when in association with Raf, ERK and MP1 and is found to be localized in cellular vesicles. The formation of this complex is selectively induced by serum and LPA stimulation of GPCRs. Interestingly, growth factors signalling through RTKs do not induce the formation of this complex (Kolch 2005). A complex of MP1 and another protein, p14, as well as KSR1 can respond to both RTK and GPCR signals (Vomastek et al. 2004). The selectivity of these scaffolds and adapters allows the fine-tuning of the ERK signal, both spatially and temporally. These scaffolds and adapters act to positively regulate the ERK pathway but it is also necessary to have endogenous inhibitors of the pathway.

1.4.4 RKIP

Ras kinase inhibitor protein (RKIP) is an endogenous inhibitor of the Raf/MEK/ERK pathway. In unstimulated cells it acts by binding to both Raf and MEK and thus preventing their physical interaction (Yeung et al. 1999). It may also be involved in reducing the activation of Raf itself by preventing its phosphorylation by PAK, as discussed previously. Upon stimulation of a GPCR, protein kinase C phosphorylates RKIP causing it to dissociate from Raf. RKIP can then bind to GRK2, a protein that usually inhibits the activation of GPCRs, disabling this inhibitory activity (Lorenz et al. 2003). Therefore, this single phosphorylation event on RKIP not only allows for activation of MEK by ERK but also removes a second inhibitor of the pathway in GRK2.

1.5 S1P signalling in astrocytes: The role of ERK

S1P stimulation of astrocytes has been shown to induce both their migration (Mullershausen et al. 2007) and their proliferation (Pébay et al. 2001; Sorensen et al. 2003). This proliferation is ERK dependent and may be regulated through S1PR2 or S1PR3s as pFTY720 and specific S1PR1 agonists have been shown to increase the migration of astrocytes without increasing their overall number (Mullershausen et al. 2007). However, S1PR1 activation has been shown to induce the phosphorylation of ERK in a dose-dependent manner. A study carried out by Osinde et al. (2007)

demonstrated that S1P induces a significant increase in the levels of pERK seen in mixed cortical and enriched astrocyte cultures compared to untreated controls. pFTY720 also induces ERK phosphorylation and does so with approximately ten times the potency of S1P (Osinde et al. 2007). These phosphorylation events were then shown to be sensitive to both MEK inhibition and pertussis toxin treatment indicating that the classical ERK pathway is recruited through a G_i coupled receptor, likely S1PR1 although possibly S1P3 (Osinde et al. 2007). The likelihood of S1PR1 being the primary receptor- type involved in ERK activation in astrocytes was further confirmed when the effects of selective S1PR1 agonists, SEW2871 and AUY954, were shown to imitate those of S1P and pFTY720 (Osinde et al. 2007). Taken together, these results indicate that there is a complicated relationship between S1P signalling, ERK phosphorylation and the cellular responses this induces. S1P activates S1PR1 (G_i coupled), S1PR2 and S1P3 (G_i, G_q and $G_{12/13}$-coupled) receptors on astrocytes and promotes cell proliferation in an ERK dependent manner through one or more of these receptors. On the other hand, pFTY720, a full and potent agonist of S1PR1 with no effect on S1PR2 and partial agonism of S1PR3 induces ERK phosphorylation and cell migration in astrocytes, likely through S1PR1, but does not promote proliferation. The proliferative effects of endogenous S1P are therefore likely partially due to the activation of S1P3 and a balance between S1PR1 and S1PR2 signalling, as S1PR2 has been shown to inhibit migration (Okamoto et al. 2000) thus countering the S1PR1 signal. The different effects of pFTY720 and S1P stimulation on astrocytes appear to be linked to interactions between the various S1PR sub-types and the relative strength of their coupling to the various G-proteins. For instance, pFTY720 causes better coupling of S1PR1 to G_i-proteins than S1P, leading to more efficient signalling through those associated pathways.

1.6 Aims and hypothesis

ERK signalling is associated with G_i and G_q-protein coupled receptor activation and has been shown to be necessary for S1P induced astrocyte proliferation. However, the mechanisms involved in S1P signalling and ERK activation in astrocytes are, as yet, unclear. In this study, the effects of a small blocking peptide (MNP301) made up of the last 10 amino-acids of the C-terminus of the S1PR1 was used to modulate post-activation signalling by S1PR agonists. We have previously shown that this peptide inhibits FTY720 induced S1PR1 internalisation in astrocytes as well as S1PR1 induced Ca^{2+} signalling (Healy et al. 2013). In addition, specific S1PR1 agonists and antagonist were used to isolate what the relative involvement of S1PR1 and S1PR3s are in these ERK phosphorylation events.

The hypothesis was that MNP301 would inhibit intracellular signalling between S1PR1 and its downstream binding partners and to reduce ERK phosphorylation. The effects of MNP301 were proposed to be similar to those of S1PR1 antagonists that inhibit ERK activation by blocking S1PR1 activation.

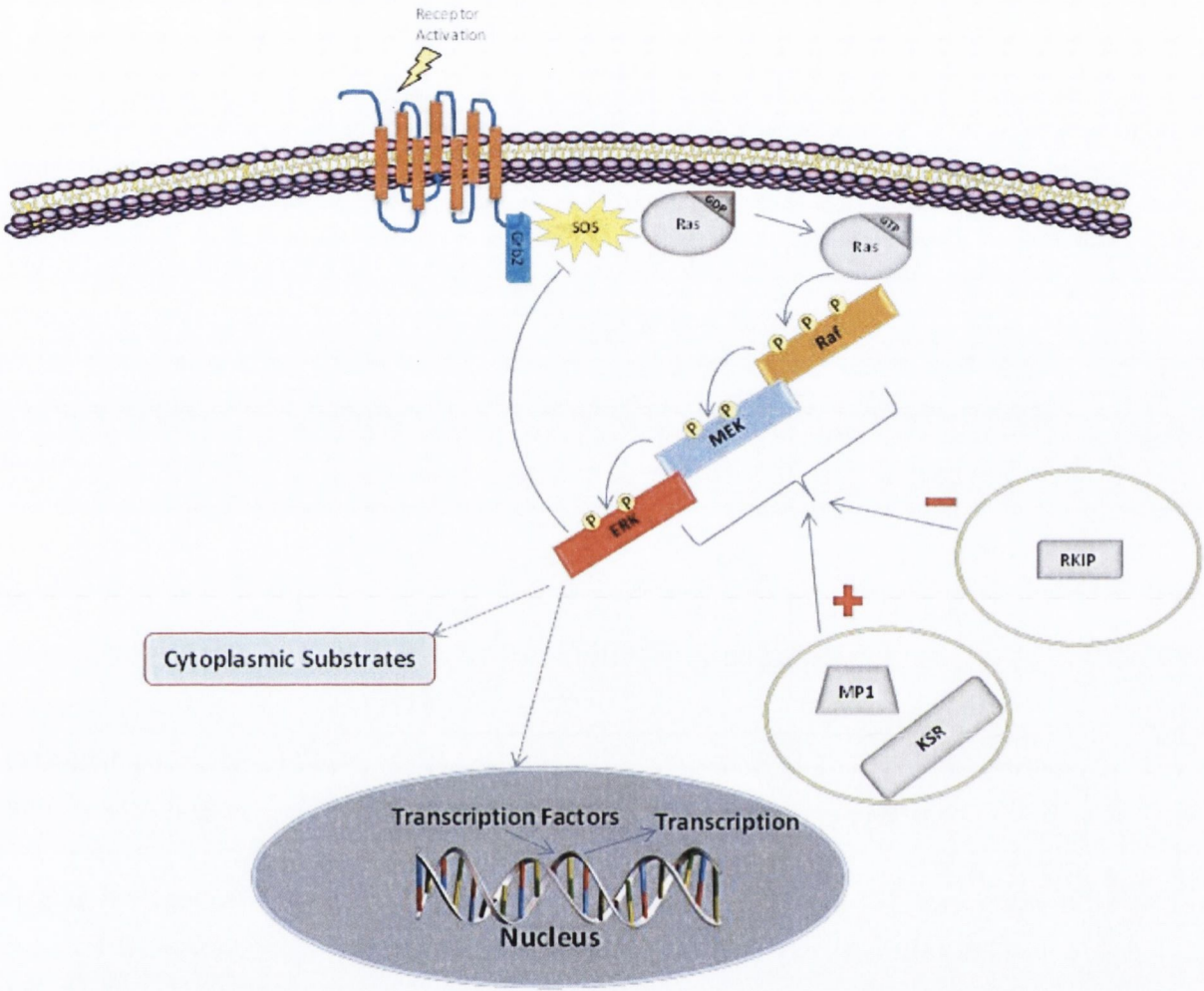

Figure 3.1 Schematic of the GPCR induced activation of ERK

GPCRS activate son of sevenless (SOS) which converts Ras from its inactive GDP bound form to its active GTP bound state. This activates a member of the Raf protein family which in turn activates MEK and subsequently ERK. ERK activates numerous cytoplasmic substrates and regulates the activity of various transcription factors.

2 Results

2.1 S1PR1 agonist AUY954 induces ERK phosphorylation in a time and concentration-dependent manner in rat astrocytes.

In order to elucidate the kinetics of S1PR1 activation of ERK, a dose and time response study for AUY954, an S1PR agonist, was conducted. ERK phosphorylation via S1PRs is generally a transient occurrence and maximal responses can be seen between 10 and 30 minutes of treatment (Osinde et al., 2007). In an assay system that can only examine the effects of treatment in each sample at an isolated time-point (namely Western blotting) it is important to ascertain the time post-treatment at which phosphorylation is approximately at its peak. In this regard, a time-course ranging from 0 to 60 minutes was examined and it was found that the highest levels of AUY954 (10nM) induced pERK can be seen at 10 minutes post-treatment. In addition, a concentration-curve examined at 10 minutes post-treatment indicates that a maximal ERK phosphorylation response occurs between 10 and 100nM (**Figure 3.2**).

2.2 S1PR1 antagonist BYR185 inhibits S1PR1 induced ERK phosphorylation in rat astrocytes

S1P and pFTY720 activate both S1PR1 and S1PR3 on astrocytes and induce the phosphorylation of ERK via one or both of these receptors. This phosphorylation of ERK has been proposed to be integral to the S1P-induced proliferation of mouse astrocytes (Pebay et al., 2001) as upstream inhibition of ERK phosphorylation attenuated S1P induced proliferation. It is possible to examine whether S1PR1, S1PR3 or both are necessary for ERK phosphorylation through the use of the S1PR1 specific agonist AUY954 and the S1PR1 specific antagonist, BYR185. AUY954 (10nM) (221.5 ± 20.6% SEM) causes a similar increase in the level of ERK phosphorylation to that seen with both S1P (100nM) (253.1 ± 21.6% SEM) and pFTY720 (10nM) (260.9 ± 33.4% SEM) treatments when compared to control (**Figure 3.3**). While both S1P and pFTY720 treated samples display a modest decrease in pERK when pre-treated with S1PR1 specific antagonist, BYR185 (203.3 ± 11.4% and 231.9 ± 48.6% SEM of untreated control respectively), the levels of pERK seen in the BYR185 pre-treated and subsequent AUY954 treated samples were no different to those seen in untreated controls (100.5 ± 22.8% SEM) (**Figure 3.3**). No difference between ERK1 and ERK2 phosphorylation was seen with any treatment and therefore quantification was performed by combining both bands to get an overall pERK or total ERK signal. The effects of specific S1PR1 antagonism by BYR185 on pFTY720/S1P induced pERK and that seen with S1PR1 specific agonist, AUY954, indicates that, while S1PR1

induces significant ERK activation, there is also the potential for activation through the S1PR3 that can compensate for the attenuation of the S1PR1signal.

2.3 Pan-S1P receptor activation on rat astrocytes induces ERK phosphorylation and is unaffected by the peptide MNP301

Work published by Healy et al. (2013) demonstrates that pFTY720 significantly impairs the induction of cAMP by forskolin. Furthermore, the attenuation of this pathway is unaffected by pre-treatment with MNP301, a peptide that mimics the last 10 residues of the S1PR1 C-terminus with an additional TAT-tag and is thought to be a pathway specific S1PR1 inhibitor. However, calcium signalling induced by activation of S1PR1 by S1P and also the S1PR1 agonist AUY954 is potently inhibited by this peptide. This data indicates that upon S1PR1 activation, MNP301 inhibits specific pathways while leaving others unaffected. This ability to modulate the activation of downstream signalling molecules without antagonising all activation of the S1PR1 could potentially be a useful tool in the examination of S1PR1 function in astrocytes. To further explore the potential of MNP301 as a pathway specific inhibitor, the effects of the peptide on ERK phosphorylation were examined. Studies conducted by Osinde et al (2007) showed that pFTY720 and S1P both transiently activated the ERK signalling pathway with pFTY720 being approximately ten times more potent in this regard than S1P. In accordance with this study, the relative pERK content of rat astrocyte samples, treated with 10nM pFTY720 or 100nM S1P for ten minutes was examined by Western blots. It was shown that both pFTY720 (296 ± 14% SEM) and S1P (304 ± 12% SEM) induce a significant increase in ERK phosphorylation ($p<0.001$ based on one-way ANOVA and Newman-Keul's post-hoc test) (**Figure 3.4**). The levels of ERK phosphorylation are unaffected by pre-treatment with 100µg/ml MNP301 (Effective concentration as published by Healy et al. 2013). While Osinde et al. (2007) proposed that the S1PR1 is the primary S1P receptor subtype involved in the induction of ERK phosphorylation, both S1P and pFTY720, also activate S1PR3 on astrocytes and the potential involvement of these S1PR3s in the activation of the ERK signalling cascade was then examined.

2.4 The peptide MNP301 significantly reduces S1PR1 induced ERK phosphorylation in astrocytes

Data previously described in this study has shown that S1PR3 activation can significantly contribute to the phosphorylation of ERK in astrocytes. MNP301 mimics the extreme C-terminus of S1PR1 but has no homology for other S1PRs. Therefore, it

is proposed that MNP301 is an S1PR1-specific modulator. The lack of efficacy of MNP301 in S1P and pFTY720 induced pERK may be due to the S1PR3 contribution to the activation of ERK. This S1PR3-activation may have masked any effects the peptide had on the S1PR1 component of ERK phosphorylation induced by S1P or pFTY720, as was seen when cells were pre-treated with BYR185. In order to isolate the effects of MNP301 on S1PR1 induced ERK phosphorylation, cells were pre-treated with 100µg/ml of the peptide before being treated with AUY954 (10nM) for 10 minutes. In these experiments MNP301 significantly blocked AUY954-mediated induction of pERK down to control levels (255 ± 25% vs. 130 ± 25% SEM) but, as previously seen, had no effect on pFTY720 induced pERK (**Figure 3.5**). These data showed that MNP301 prevented pERK signalling induced by selective S1PR1 compounds (such as AUY954), but not by pan-S1PR compounds (such as S1P or pFTY720) (**Figure 3.5**).

2.5 MNP301 does not affect S1PR independent ERK phosphorylation

MNP301 has been shown to reduce the phosphorylation of ERK induced through S1PR1 on astrocytes but not other S1PRs. Despite a lack of homology between the MNP301 amino-acid sequence and protein sequences other than S1PR1, there is a possibility that MNP301 could interfere with ERK phosphorylation induced by other receptors. The possibility that the peptide may interfere with the phosphorylation of ERK induced by other GPCRs, unrelated to S1PRs, was therefore also explored. In order to examine to corroborate the specificity of MNP301 for S1PR1-activated pERK, astrocytes were treated with glutamate (30µM) after a 1hour MNP301 treatment. Glutamate induced significant activation of ERK signalling (205.4 ± 5.6% SEM of control level) ($p<0.01$ based on one-way ANOVA and Newman-Keuls post-hoc test) (**Figure 3.6**) that was unaffected by the presence of the MNP301 blocking peptide (180.2 ± 4.5% SEM) ($p<0.05$ based on one-way ANOVA and Newman-Keuls post-hoc test) (**Figure 3.6**). These data support the theory that MNP301 modulates the activation of specific signalling pathways associated with S1PR1 but not other S1PRs or unrelated GPCRs.

2.6 Pretreatment with AUY954 attenuates subsequent ERK phosphorylation by AUY954 and is unaffected by MNP301

Receptor internalisation is one of the key mechanisms by which S1PR ligands are thought to be efficacious (Gräler & Goetzl 2004; Thangada et al. 2010). In the work published by Healy et al. (2013) MNP301 is shown to prevent S1PR1 internalisation induced by pFTY720. Work by Pan et al (2006) showed that AUY954 also brings about

significant internalisation of the S1PR1. The internalisation of S1PR1 leaves the cell unable to respond to any other agonists present in the cellular environment. In the current study, after a 1 hour treatment with AUY954, astrocytes cannot respond to an additional acute 10 minute treatment with the agonist and no increase in pERK is seen when compared to control or 1 hour treatments (**Figure 3.7**). It was proposed that MNP301 would inhibit AUY954 induced internalization of S1PR1, thereby allowing the receptor to respond to the acute 10 minute treatment despite the 1 hour pre-treatment with AUY954. This was not found to be the case as no significant increase in pERK was seen when an initial 1 hour treatment of AUY954, in the presence of MNP301, was followed by a further 10 minute treatment (**Figure 3.7**). Taken together, these data indicate that despite the fact that Healy et al. (2013) demonstrated that MNP301 prevents the internalisation of S1PR1, MNP301 does affect the inhibition of ERK phosphorylation upon pre-treatment with AUY954 prior to acute 10 minute treatment. The lack of effect of MNP301 in this experiment may be unrelated to any effect on internalisation but may rather be due to the inhibition of pERK induced by MNP301 as previously described.

2.7 Inhibition of Ca^{2+} signalling does not inhibit S1PR1-induced ERK phosphorylation

Ligand binding to S1PR1 recruits multiple intracellular signalling molecules including cAMP, ERK and calcium. There is evidence that elevation of calcium may be linked to the regulation of the ERK signalling cascade (Chuderland and Seger, 2008). This is pertinent as MNP301 has been shown to interfere with the increase of calcium associated with S1PR1 activation (Healy et al 2013). The MNP301 mediated inhibition of pERK as described previously could possibly be related to this effect of the peptide on calcium signalling events. To explore this possible interaction, two inhibitors of calcium signalling EGTA and dantrolene, were used. EGTA is a chelator of calcium, among other ions, and inhibits extracellular calcium whereas dantrolene is an inhibitor of ryanodine receptor, linked to intracellular stores. Neither dantrolene nor EGTA displayed any influence on basal pERK levels (77.2 ± 4.25% and 96.88 ± 7.9% SEM of control respectively) and had no significant effect on AUY954 (182.9 ± 26.2% SEM of control) induced increases in pERK (175.9 ± 24.8% and 159.2 ± 13.75% SEM of control respectively) (**Figure 3.8**). Taken together, this indicates that the impact of MNP301 on pERK is not linked to its influence on calcium signalling as inhibition of calcium signalling does not impact on S1PR1 activated pERK.

2.8 Identification of active site in MNP301

MNP301 is a ten amino acid long peptide chain that mimics the extreme C-terminus of S1PR1. This sequence of amino acids contains a serine-rich region that has been proposed to be essential for the internalisation dynamics of the receptor after ligand binding (Liu et al 1999, Oo et al 2007, Watterson et al 2002). To examine which amino acids were necessary to prevent the phosphorylation of ERK induced by AUY954 three mutants of the original MNP301 peptide were created. These peptides substituted a triple alanine region for amino acids in the wild-type sequence. MNP301=YGRKKRRQRRR-MSSGNVNSSS, MNP302=YGRKKRRQRRR-MSSG NVNAAA, MNP303=YGRKKRRQRRR-MSSGAAASSS, MNP304=YGRKKRRQRRR-MAAANVNSSS. It was found that MNP302 (103.3 ± 11.62% SEM of control) reduced pERK induction by AUY954 (142.3 ± 8.2% SEM) while the other peptide sequences, MNP303 and MNP304 were ineffective (142.6 ± 18.55% and 141.7 ± 25.3% SEM respectively) (**Figure 3.9**). This data implies that the final three serine molecules of the S1PR1 C-terminus are not integral to the induction of ERK phosphorylation and the site of interaction between the C-terminus and the interacting proteins in question are located before this extreme C-terminus serine region.

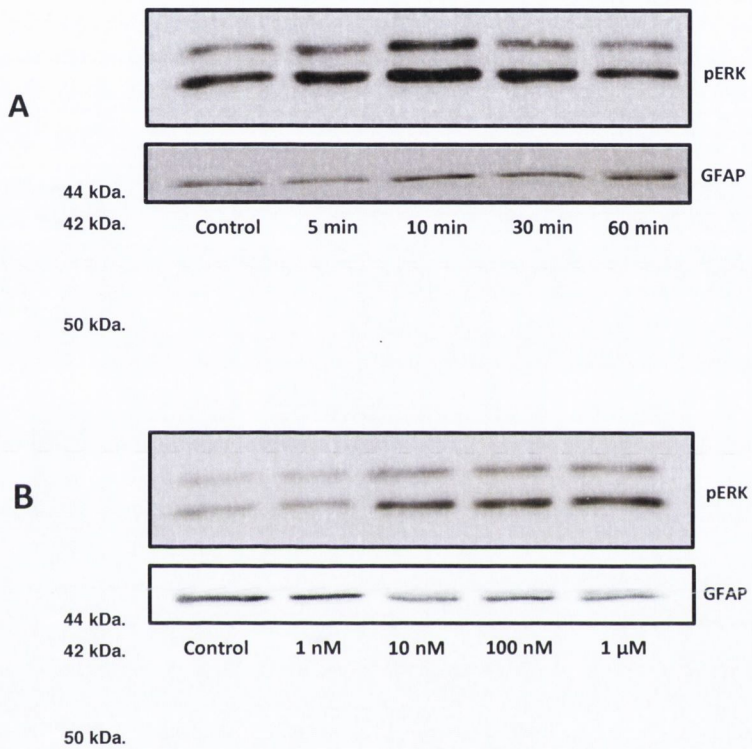

Figure 3.2 S1PR1 agonist AUY954 induced ERK phosphorylation is time and concentration-dependent in astrocytes

(A) Rat astrocytes were serum starved for 3hr and treated with 10nM AUY954 for 1-60 min, after which cells were scraped and processed for Western blotting. **(B)** Cells were serum starved for 3hr and treated with 1nM-1µM AUY954 for 10 min, after which cells were scraped and processed for Western blotting. (N=4 independent experiments)

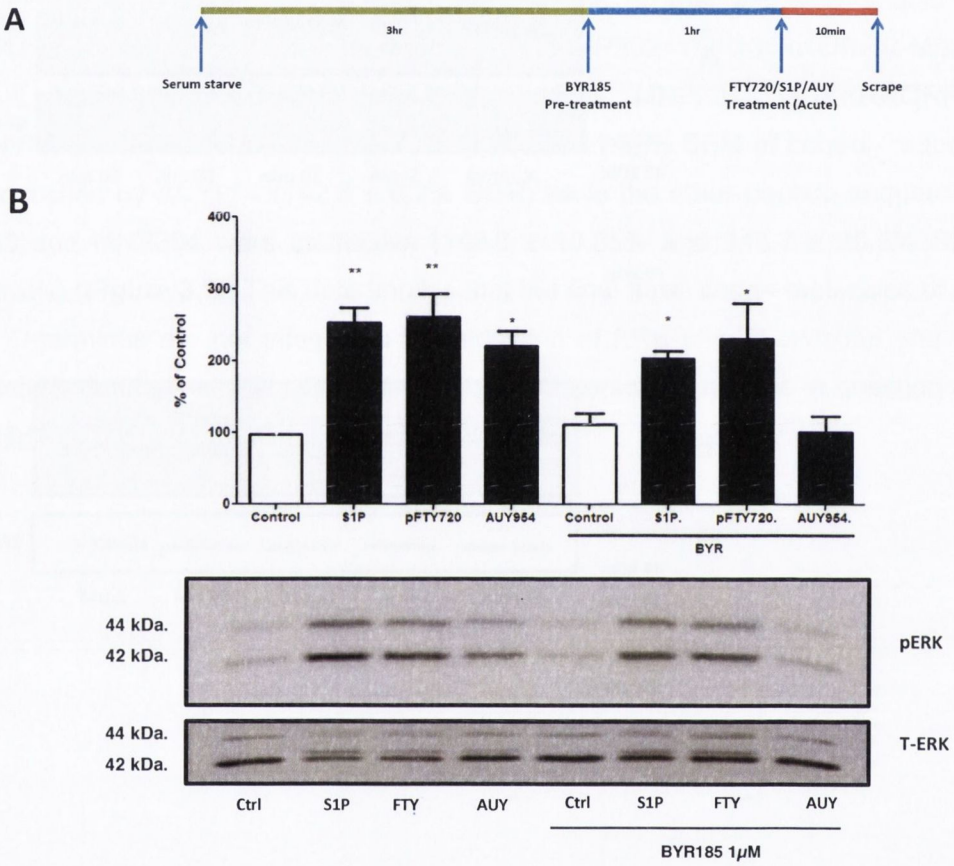

Figure 3.3 S1PR1 antagonist BYR185 inhibits S1PR1 induced ERK phosphorylation in rat astrocytes

(A) Rat astrocytes were serum starved for 3h, pre-incubated with BYR185 (1μM) for 1h and then treated with S1PR ligands for 10 min (S1P 100nM, pFTY720 10nM, AUY954 10nM) after which cells were scraped and processed for Western blotting. **(B)** Pre-treatment of cells with BYR185 ablates ERK phosphorylation induced by AUY954 but not S1P or pFTY720. The bands from both (p)ERK isoforms were quantified together as an overall (p)ERK signal. ***p<0.001, **p<0.01, *p<0.05 significant difference compared to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM (N=5 independent experiments).

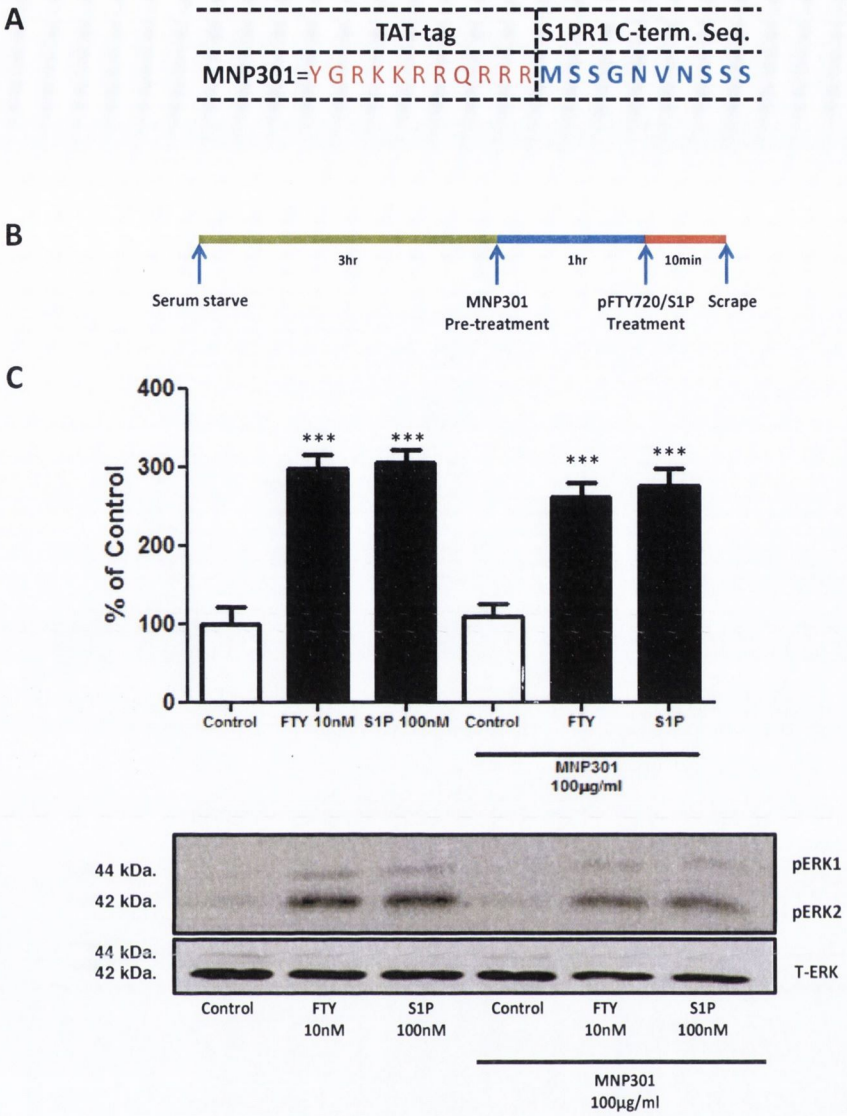

Figure 3.4 Pan-S1P receptor activation induces ERK phosphorylation and is unaffected by the peptide MNP301

(A) The amino-acid sequence of MNP301 mimics the last 10 residues of the S1PR1 C-terminus with an additional TAT-tag. **(B)** Rat astrocytes were serum starved for 3h, pre-incubated with MNP301 (100µg/ml) for 1h and then treated with S1PR ligands (S1P 100nM, pFTY720 10nM) for 10 min, after which cells were scraped and processed for Western blotting. **(C)** ERK phosphorylation induced by pFTY720 and S1P is not significantly reduced by pre-treatment with MNP301. ***p<0.001, significant difference compared to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM (N=3 independent experiments).

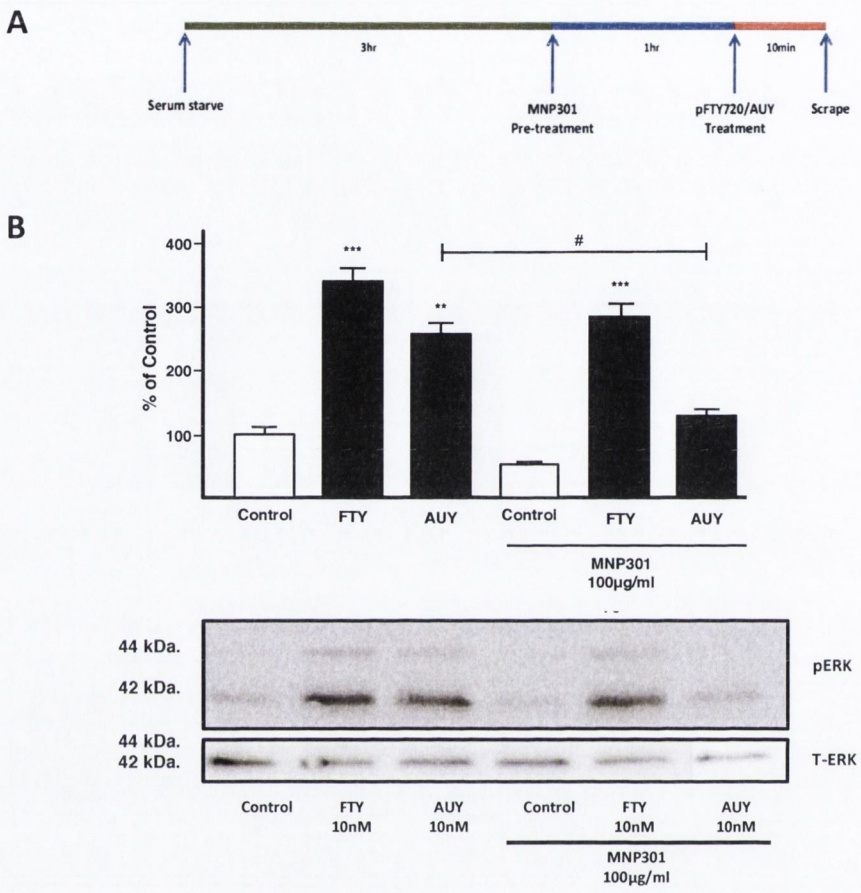

Figure 3.5 MNP301 significantly reduces S1PR1 induced ERK phosphorylation.

(A) Rat astrocytes were serum starved for 3h, pre-incubated with MNP301 (100µg/ml) for 1h and then treated with S1PR ligands (10nM for both) for 10 min, after which cells were scraped and processed for Western blotting. **(B)** ERK phosphorylation induced by AUY954 is significantly reduced by pre-treatment with MNP301. ***$p<0.001$, **$p<0.01$ significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. #$p<0.05$ significant difference between AUY and AUY+MNP301 treated samples based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM (N=4 independent experiments).

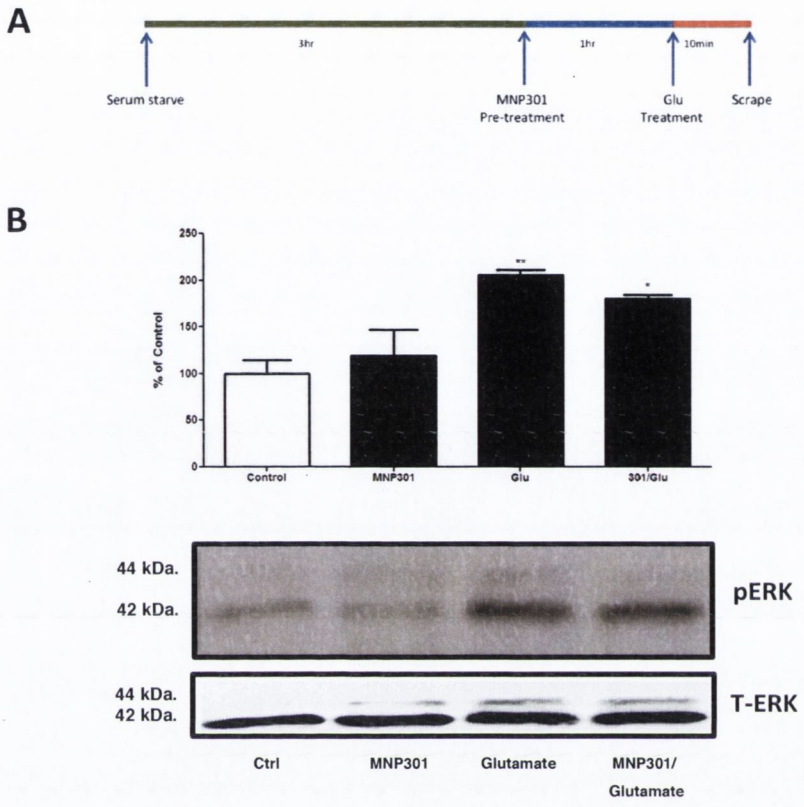

Figure 3.6 MNP301 does not affect S1PR independent ERK phosphorylation.

(A) Rat astrocytes were serum starved for 3h, pre-incubated with MNP301 (100µg/ml) for 1h and then treated with glutamate (Glu) (30µM) for 10 min, after which cells were scraped and processed for Western blotting.**(B)** ERK phosphorylation induced by glutamate is unaffected by pre-treatment with MNP301. **p<0.01, *p<0.05 significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM (N=4 independent experiments).

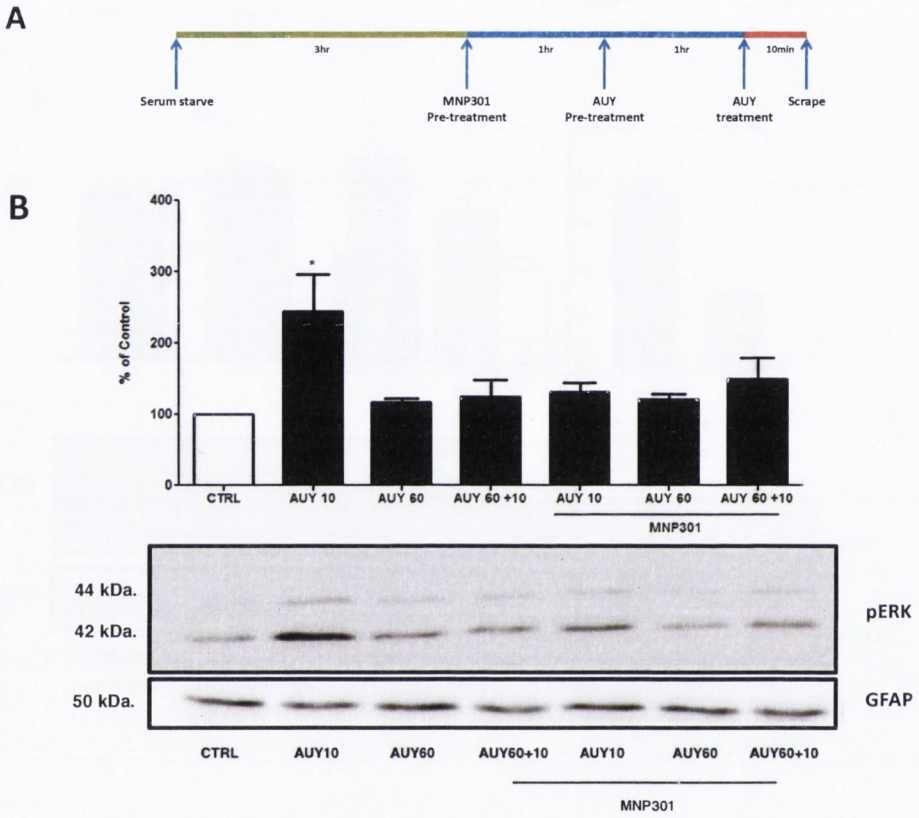

Figure 3.7 Pretreatment with AUY954 attenuates subsequent ERK phosphorylation by AUY954 and is unaffected by MNP301

(A) Rat astrocytes were serum starved for 3h, pre-incubated with MNP301 (100µg/ml) for 1h and then treated with AUY954 (10nM) for 1 hour followed by a further 10 minute treatment, after which cells were scraped and processed for Western blotting.**(B)** AUY954 pre-treatment prevents further phosphorylation of ERK that is unaffected by MNP301. *$p<0.05$ significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM. (N=3 independent experiments).

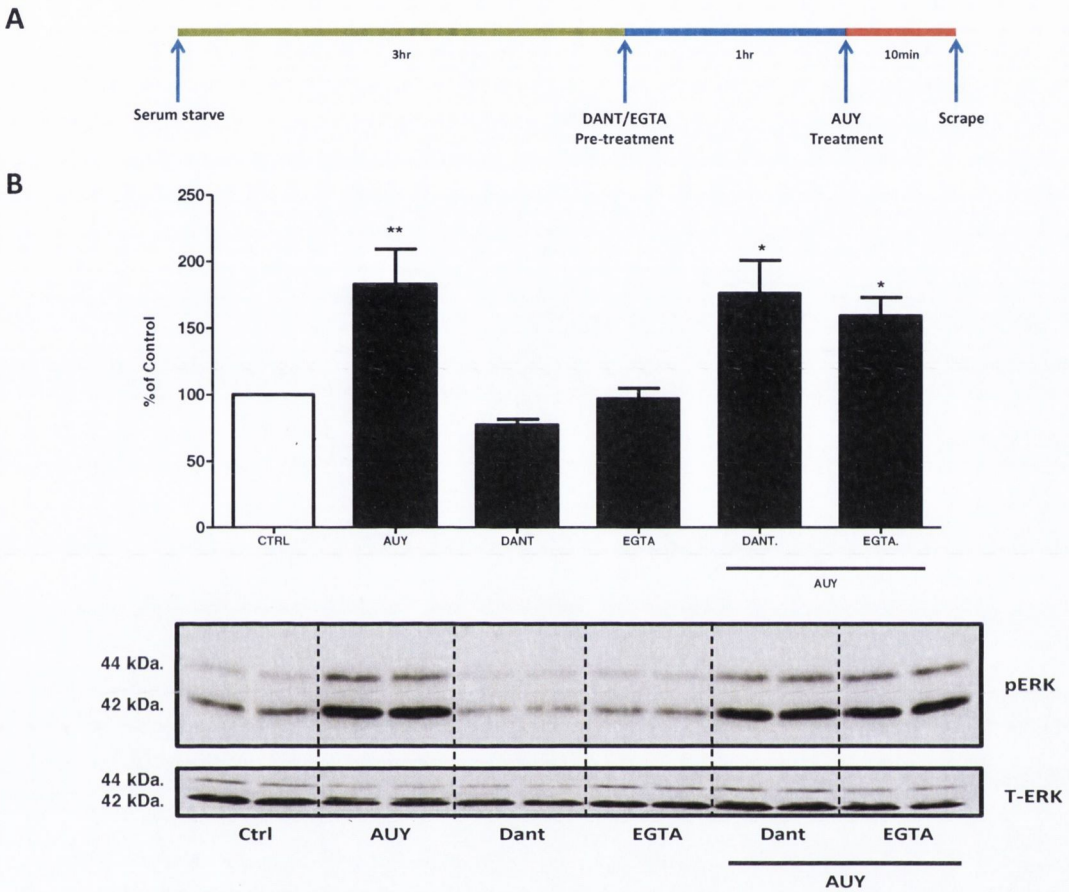

Figure 3.8 Inhibition of Ca^{2+} signalling does not inhibit S1PR1-induced ERK phosphorylation

(A) Rat astrocytes were serum starved for 3h, pre-incubated with EGTA (1mM) or dantrolene (30μM) for 1h and then treated with AUY954 (10nM) for 10min. **(B)** Pre-treatment of cells with dantrolene (30μM) or EGTA (1mM) had no effect on ERK phosphorylation induced by AUY954. **p<0.01, *p<0.05 significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM (N=4 independent experiments).

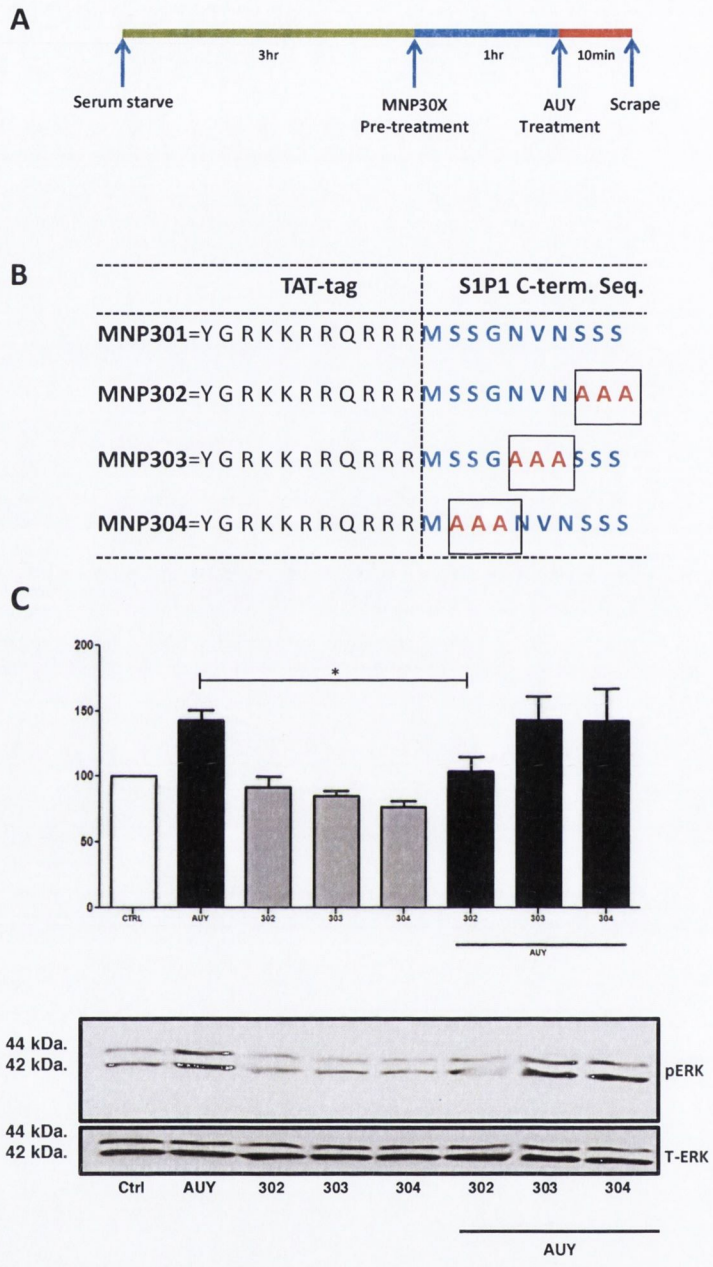

Figure 3.9 Identification of active site in MNP301

(A) Rat astrocytes were serum starved for 3h, pre-incubated with MNP30X peptides (all at 100µg/ml) for 1h and then treated with AUY954 (10nM) for 10 min, after which cells were scraped and processed for Western blotting. **(B)** Amino-acid sequences of mutated MNP30X peptides. **(C)** Pre-treatment of cells with MNP302 reduced ERK phosphorylation induced by AUY954. No other MNP30X peptide showed any efficacy in reducing ERK phosphorylation. * $p<0.05$ based on student T-test comparing AUY to MNP302+AUY. Graph depicts mean ± SEM (N=4 independent experiments)

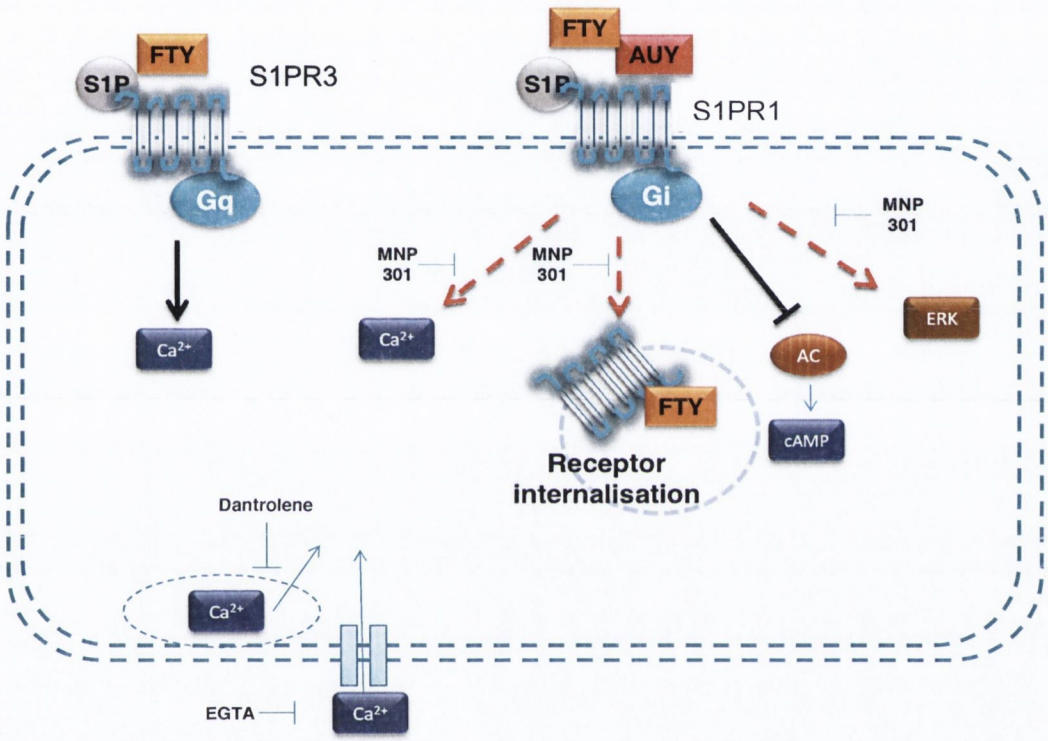

Figure 3.10 Proposed effects of MNP301 in astrocytes.

It has been established that the peptide MNP301 inhibits FTY720-induced S1PR1-internalisation in astrocytes. In addition MNP301 also inhibits S1PR1-associated calcium signalling induced by FTY720, S1P and AUY954, but has no effect on S1PR3-mediated Ca^{2+} signalling (Healy et al. 2013). This current study demonstrates that MNP301 also inhibits ERK phosphorylation via S1PR1 through an, as yet, unidentified mechanism.

3 Discussion

3.1 Is the phosphorylation of ERK solely regulated by S1PR1?

Work by Osinde et al. (2007) proposed that the primary S1P receptor involved in the activation of the ERK signalling pathway in astrocytes was S1PR1. This assumption was based on the fact that the S1PR1-agonist SEW2871 induced a significant level of pERK, in line with that seen by S1P and pFTY720, and that specific antagonists of S1PR1 reduced these levels of phosphorylation. However, the S1PR1 antagonist did not attenuate S1P induced ERK phosphorylation indicating that an one or more of the other S1PRs present on astrocytes can induce the phosphorylation of ERK (Osinde et al. 2007). Further, recent work by Fischer et al. (2011) has shown that treatment of astrocytes with a specific S1P3 agonist, named compound 20, induces significant activation of ERK (Fischer et al. 2011). Both S1P and pFTY720 activate S1PR1 and S1PR3s on astrocytes and therefore any inhibitory effect of the peptide may be compensated for by the activation of G_i or G_q-coupled S1PR3s. This current study aimed to examine the role of S1PR-mediated pERK activity in primary rat astrocyte cultures. It was found that both S1P (100nM), pFTY720 (10nM) and AUY954 (10nM) brought about a significant increase in the levels of pERK seen after 10 minutes incubation with the compounds. Furthermore, pre-treatment with the novel S1PR1-specific antagonist BYR185 attenuated levels of pERK seen only in AUY954 treated samples. BYR185 pretreatment followed by S1P or pFTY720 treatments still displayed a significantly elevated level of pERK when compared to untreated controls. These data demonstrate that S1PR activation can induce pERK in the absence of S1PR1 activity and the likelihood is that this involves agonism of S1PR3. It is difficult to establish the importance of S1PR3 signals as there is a lack of potent, specific S1PR3 antagonists.

3.2 Does MNP301 regulate S1PR-mediated phosphorylation of ERK?

MNP301 is a ten amino-acid sequence that mimics the final amino acids of the extreme C-terminus of S1PR1. It is proposed to act as a receptor-specific and pathway-selective modulator of intracellular signalling (Healy et al. 2013). Previous studies carried out in this lab demonstrated that a FITC-tagged version of the MNP301 peptide permeates cells in an astrocyte culture in a dose-dependent manner and is visible up to 2 days post-treatment in organotypic slice cultures (Healy et al. 2013). The MNP301 peptide was further shown to reduce calcium signalling in S1P and pFTY720 treated astrocytes while attenuating calcium response seen in AUY954 treated cells (Healy et al. 2013). This is an indication that MNP301 inhibits the S1PR1-

related component of a response that is mediated through both S1PR1 and S1PR3. However, MNP301 was shown to have no effect on pFTY720 induced reduction of cAMP, although an S1PR1 selective agonist was not examined in this experiment (Healy et al., 2013). Based on these results it was unknown whether the effects of MNP301 were restricted to inhibition of calcium signalling or whether the peptide could modulate other intracellular signalling events. Here, this question was explored by an analysis of the peptide's effects on pERK. We initially found that MNP301 had no significant effect on the activation of pERK as induced by pFTY720 and S1P. However, the S1PR1 specific antagonist BYR185 did not attenuate ERK phosphorylation induced by these pan-S1PR agonists, S1P and pFTY720, while ablating the pERK signal induced by S1PR1 specific agonist AUY954. This indicated that apart from S1PR1, an additional S1PR subtype, likely S1PR3, contributes to the activation of ERK in astrocytes. This is in agreement with the work of Fischer et al. (2012). To establish if MNP301 inhibits pERK induced through specific S1PR1 activation, a comparison was made between pFTY720 and AUY954 induced pERK. It was ascertained that pERK induced through the activation of S1PR1 by AUY954 could be attenuated by MNP301 (published findings in Healy et al. 2013). These results are in accordance with the hypothesis that MNP301 is a receptor specific (regulating S1PR1 activity only) and pathway-selective (reduces calcium and pERK but has no effect on cAMP) modulator of intracellular signalling.

3.3 Is MNP301 a specific and selective modulator of S1PR1 signalling?

The selectivity of MNP301 for S1PR1 as opposed to S1PR3-mediated interactions has been consistently demonstrated in relation to calcium signalling and ERK phosphorylation. However, due to the novelty of the proposed mechanism, further confirmation of specificity was required. Two pieces of data required further exploration: firstly, that the regulation of ERK phosphorylation by MNP301 was specific to S1PR1-induced activation and the peptide was not a general inhibitor of pERK, and, secondly, that the influence of MNP301 on calcium signalling was not linked to the influence of the peptide on pERK. To address the first of these questions, the effects of MNP301 on glutamate induced phosphorylation of ERK was examined. It was found that MNP301 had no effect on the glutamate-induced increase in the levels of pERK. A possible link between the modulation of calcium signalling by MNP301 and the inhibition of pERK was then explored. Links have been described between calcium signalling and the initiation of the ERK pathway (Chuderland & Seger 2008). Calcium signalling events, as described by Healy et al. (2013), occur approx. 30 seconds post-treatment with an S1PR agonist while S1PR-mediated ERK phosphorylation peaks at

approximately 10 minutes post-activation. Therefore, temporally, it is possible that intracellular calcium could activate the ERK pathway. To examine a possible link between calcium signalling and the initiation of ERK signalling two compounds were utilised, namely ethyleneglycoltetraacetic acid (EGTA) and dantrolene. EGTA is a chelating agent similar to ethylenediaminetetraacetic acid (EDTA) but with a greater affinity for calcium ions, while dantrolene is an inhibitor of ryanodine receptors that prevents the release of calcium from intracellular stores. Neither of these agents had any effect on the phosphorylation of ERK by AUY954, indicating that S1PR1-mediated calcium and ERK signalling are regulated through separate mechanisms. These results support the hypothesis that MNP301 is receptor specific, due to a lack of effect on glutamate induced pERK, and that is a modulator of multiple pathways, not just calcium release.

3.4 Identification of the active site in MNP301

The extreme C-terminal sequence of S1PR1 contains a serine-rich region that has been proposed to be essential for the internalisation dynamics of the receptor after ligand binding (Liu et al 1999, Oo et al 2007, Watterson et al 2002). MNP301 mimics the final ten-amino acid sequence of S1PR1 and thus contains a pair and trio of neighbouring serine residues that were suspected to be important for the described effects of the peptide. Three additional peptides, named MNP302-304, were generated in order to investigate the relative importance of different sets of amino-acids within the extreme C-terminal sequence of S1PR1 (sequences are listed in **Figure 3.9**). In the case of MNP302 (YGRKKRRQRRR-MSSGNVNAAA), mutation of the three serine residues that replicate the three final amino acids of S1PR1's C-terminus to alanine residues did not impair the ability of this peptide to reduce ERK phosphorylation. However, both MNP303 (YGRKKRRQRRR-MSSGAAASSS) and MNP304 (YGRKKRRQRRR-MAAANVNSSS) lost the inhibitory ability of MNP301 when alanine residues were substituted further up the amino-acid chain. These results indicate that the final 3 amino acids of MNP301 are not involved in the inhibition of pERK and that the site of interaction is located within the remaining seven amino-acids (MSSGNVN), two of which are a serine pair. Mutation of these amino-acid residues may also induce a conformational change in the peptide that could possibly prevent interactions with other proteins, interactions that may account for the efficacy of the wild-type MNP301 sequence. Furthermore, conformational changes may influence the ability of the peptides to permeate cell membranes, preventing the mutated peptides interacting with intracellular signalling molecules. Further studies are required to examine these possibilities and to establish if other signalling pathways are affected by MNP301.

3.5 Conclusion

MNP301 has been shown to be receptor specific and pathway selective in regulating signalling events in astrocytes *in vitro*. The peptide inhibits ERK phosphorylation induced through S1PR1 specifically but not pERK induced by other GPCRs. This effect is not dependent on Ca^{2+} signalling, another signalling pathway that MNP301 has been shown to regulate, indicating that the peptide may inhibit a particular activation event upstream of ERK phosphorylation. These characteristics of MNP301 warrant further exploration. It remains to be seen what additional pathways the peptide modulates and what functional effects of the peptide can be established *in vitro.* Studies identifying additional signalling pathways modulated by the peptide as well as establishing functional outcomes *in vitro* will lead to a better understanding of the mechanism of action of the peptide. As of yet, it is not known whether the *in vitro* effects of MNP301 described result in functional outcomes and whether the peptide is stable *in vivo*. These are important questions to address, however, regardless of whether or not MNP301 is utilised *in vivo,* the development of MNP301 as an *in vitro* research tool will help the understanding of S1PR1 signalling which may in turn aid the development and refinement of clinical therapies.

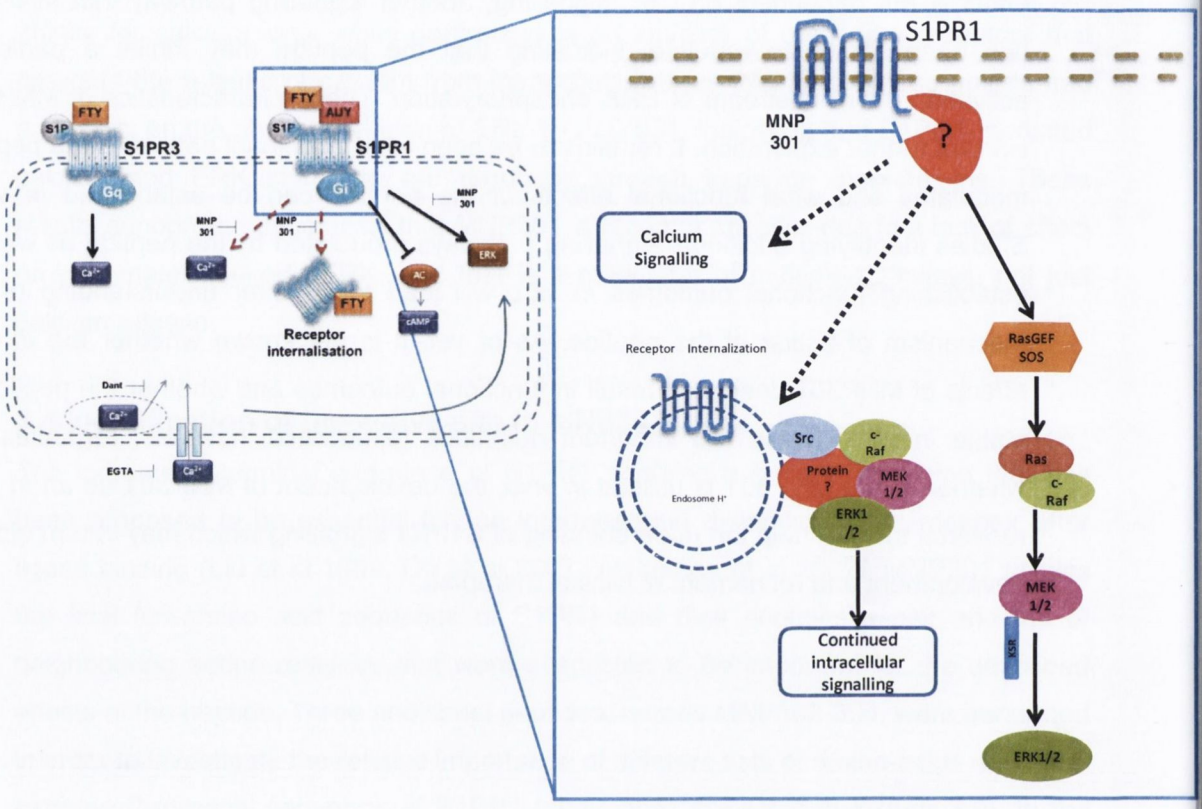

Figure 3.11 The mechanism of MNP301 inhibition of pERK is unknown

It has been established that MNP301 inhibits S1PR1 induced calcium signalling in astrocytes while leaving cAMP unaffected (Healy et al. 2013). This current study demonstrates that MNP301 also inhibits ERK phosphorylation via an, as yet, unidentified mechanism. MNP301 was designed to mimic the extreme C-terminus of the S1PR1 and thus may interfere with one or more protein-protein interactions that require the final amino-acids of the C-terminus of the receptor.

Chapter 4. The impact of S1PR1 modulation on the cross-talk of intracellular signalling molecules

Chapter Aims:

- To examine the phosphorylation of Akt as induced by S1PR1 agonism.
- To demonstrate the effects of MNP301 treatment on this S1PR1-induced Akt phosphorylation.
- To describe the mechanism through which MNP301 modulates S1PR1-induced pAkt.
- To assess the effects of S1PR1 activation in an astrocyte migration assay.
- To isolate the signalling events involved in regulation of S1PR1-induced astrocyte migration.
- To observe the effects of ERK inhibition/ Akt activation on astrocyte migration.

Abstract

Akt is a protein kinase that is linked to cell survival, protein synthesis and migration of many cell types. The dysregulated activity of phosphorylated Akt has been linked to certain types of cancers due to improper survival signalling. The phosphorylation of ERK and its upstream activators has also been the subject of research in regard to their role in the development of tumours. In the course of the cited research it has been found that cross-talk between the Akt and ERK pathways may provide a survival mechanism whereby inhibition of one pathway leads to increased activation of the other. In epithelial cells this cross-talk has also been suggested to regulate cellular migration. As previously described in Chapter 3, the S1PR1 modulator MNP301 inhibits the activation of ERK. In this current chapter, it is shown that upon activation of S1PR1 by the specific agonist AUY954, MNP301 induces an increased and prolonged pAkt signal in astrocytes. This effect is mimicked by the inhibition of pERK by the MEK inhibitor, U0126. The functional effects of S1PR1 activation were assessed using an astrocyte migration assay. It was found that AUY954 and pFTY720 both promoted significant astrocyte migration that was sensitive to inhibition by the specific S1PR1 antagonist BYR185. This migration was also inhibited by MK2206, a specific inhibitor of pAkt currently in clinical trials as a cancer therapy. The S1PR1-mediated migration of astrocytes was thus deemed to be dependent on Akt signalling. Furthermore, both MNP301 and U0126 promoted significant migration of astrocytes in the presence and absence of S1PR1 agonism. This migration was also sensitive to inhibition my MK2206. Therefore, it was proposed that ERK activation exerts a constitutive inhibition on the activation of Akt in astrocytes. This inhibition is removed by the addition of U0126 and MNP301 and a functional expression of this Akt phosphorylation is increased astrocyte migration.

1 Introduction

1.1 Akt phosphorylation

The phosphatidylinositol 3-kinase (PI3K) to mammalian target of rapamycin (mTOR) pathway is a key signalling cascade in the regulation of mammalian cell survival, proliferation and differentiation (Chappell et al. 2011). Akt (of which there are three isoforms, Akt1-3) is a component of this pathway that can be activated as part of a linear cascade from PI3K to mTOR but has many disparate downstream substrates of its own (Zhou et al. 2006). A brief description of the typical GPCR activation of this pathway (**Figure 4.1)** is as follows: PI3K is activated upon the binding of certain ligands to their cognate receptor. This in turn initiates a conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to PIP_3 through the addition of an additional phosphate residue, overcoming phosphatase and tensin homolog (PTEN) inhibition (Manning & Cantley 2007). Akt is subsequently recruited to the plasma membrane where PIP_3 phosphorylates it through the activity of phosphoinositide-dependent kinase-1 (PDK1) (Manning & Cantley 2007). Once Akt is phosphorylated, it can then activate mTOR complex 1 (mTORc1), which consists of mTOR in association with regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8, also known as GβL), as well as proline-rich AKT1 substrate 1 (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR) (Sengupta et al. 2010) (**Figure 4.1**). The other form of mTORc, namely mTORc2, is made up of mLST8, DEPTOR, rapamycin-insensitive companion of mTOR (RICTOR), stress-activated MAP-interacting protein (SIN1) and protein observed with RICTOR (PROTOR) and can activate AKT by directly phosphorylating serine 473 (De Luca et al. 2012). Akt has numerous downstream substrates that induce (1) protein synthesis (via mTORC1); (2) control cell survival by inhibiting FoxO1 (Rena et al. 1999) and Bad (Maiti et al. 2001); (3) enhance cell proliferation through inhibition of Wee1(Katayama et al. 2005), Myt1 (Okumura et al. 2002) and GSK-3 (Harris et al. 2001); (4) modulate glucose transport through PIP5K (Berwick et al. 2004) and AS160 (Kane et al. 2002); (5) while also regulating Ataxin-1 (Jorgensen et al. 2007), Huntingtin (Humbert et al. 2002), which are both involved in protein aggregation and neurodegeneration and (6) also regulate NF-κB signalling induced by TNF-α (Ozes et al. 1999) (**Figure 4.1**).

1.2 Akt phosphorylation and migration

In some cellular systems, the phosphorylation of Akt has been proposed to regulate migration (Irie et al. 2005; Zhou et al. 2006). Breast tissue endothelial cells rely on the complex interaction between Akt phosphorylation and the phosphorylation of ERK to

control the process of migration *in vitro* (Irie et al. 2005). In the cited study, Akt1 was shown to inhibit insulin-like growth factor 1 (IGF-IR)-induced stimulation of epithelial cell migration. In contrast, down-regulation of Akt1 promoted this migration as well as enhancing ERK1 and 2 activation, which was deemed to contribute to this migratory effect (Irie et al. 2005). Notably, the activation of Akt2 performed the opposite function and promoted epithelial cell migration, while having no effect on ERK activity (Irie et al. 2005). The mechanism by which Akt1 but not Akt2 cross-regulates ERK phosphorylation has not been described (**Figure 4.2**) and the definition of roles for Akt1 and Akt2 may differ among cell types. In this regard, Akt1 has been shown to be a promoter of embryonic fibroblast migration with Akt2 deemed to inhibit this mechanism (Zhou et al. 2006). Akt and ERK phosphorylation have also been indicated in the migration of primary rat astrocytes *in vitro* (Wang et al. 2005) (**Figure 4.2**). The activation of both of these signalling molecules was shown to be required in $P2Y_2$-receptor (P2 nucleotide receptor for adenosine tri-phosphate (ATP) and uridine 5'-triphosphate (UTP) mediated induction of astrocyte migration upon stimulation with UTP (Wang et al. 2005).

Intracellular signalling molecules typically act downstream of the receptor that initiates the activation of the particular cascade. However, in the case of S1PR1 and Akt, there is evidence to show that there is a post-activation interaction between Akt and the receptor (Lee et al. 2001). S1P induces migration of endothelial cells through S1PR1 and requires pAkt to bind to and phosphorylate the third intracellular loop of the receptor. This transactivation does not play a role in the G_i-signalling of the receptor but is necessary for Rac activation, membrane re-organisation and consequent migration (Lee et al. 2001). Mullershausen et al. (2007) studied the effects of S1PR-mediated migration of astrocytes in a purified culture using a scratch-wound assay as well as in hippocampal organotypic slice cultures. It was found that pFTY720 and the S1PR1 specific agonist SEW2871 promoted astrocyte migration in the absence of any change in the rate of proliferation compared to controls. The pathways involved in this S1PR1-mediated chemotaxis are as yet unidentified.

1.3 Cross-talk between intracellular signalling molecules.

Dysfunction of the PI3K/Akt and MAPK/ERK pathways is closely linked to many forms of cancer, as deregulated activation of these pathways allows cellular proliferation to continue unchecked. For this reason, novel clinical investigations have identified these pathways as important targets for cancer therapeutics (De Luca et al. 2012). During the course of some of these studies it has been found that despite the complete

inhibition of one of these pathways, cellular survival and proliferation is not reduced as expected (Normanno et al. 2006; Serra et al. 2011). It transpires that inhibition of the PI3K/Akt pathway may in fact lead to increased activity of the MAPK/ERK pathway, and *vice versa,* possibly through the removal of Akt-inhibition of Raf (Zimmermann & Moelling 1999) (**Figure 4.2**). In human epidermal growth factor receptor 2 (HER-2) overexpressing breast cancer cells, it was found that complete inhibition of PI3K, upstream of AKT, deleted the AKT signal but lead to an enhanced ERK phosphorylation response (Serra et al. 2011). This enhanced ERK response was sensitive to both antagonism of the HER-2 receptor and the inhibition of MEK, upstream of ERK (Serra et al. 2011). Conversely, the blockade of MAPK pathways in breast cancer cells through the use of the MEK inhibitor, PD98059, was associated with an increase in Akt phosphorylation (Normanno et al. 2006). It appears that there may be an inbuilt compensatory mechanism whereby cells respond to the inhibition of one pro-survival pathway by upregulating the activity of another. This compensatory mechanism may also influence migration as, mentioned previously, there is evidence to suggest that the activation of Akt1 can have an inhibitory effect on ERK activation and consequently diminish the levels of epithelial cell migration induced by IGF-1R activity (Irie et al. 2005) (**Figure 4.2**).

1.4 Aims and Hypothesis

Previous work has already shown that the S1PR1 activation recruits the ERK signalling pathway. Further, we have shown that MNP301, an S1PR1-specific pathway modulator, can bring about inhibition of this activation (**Chapter 3**) (Healy et al. 2013). This current chapter aims to examine the effects of S1PR1 modulation on other signalling molecules, in particular the Akt signalling pathway. In addition, the functional effects of this modulation were examined in a migration assay.

The hypothesis is that, similar to the ERK pathway, Akt phosphorylation will be modified by the addition of MNP301 in the presence of an S1PR1 agonist. Moreover, the established modulation of S1PR1-induced pERK by MNP301 and/or putative effects on pAkt will influence the relative levels of migration of rat astrocytes.

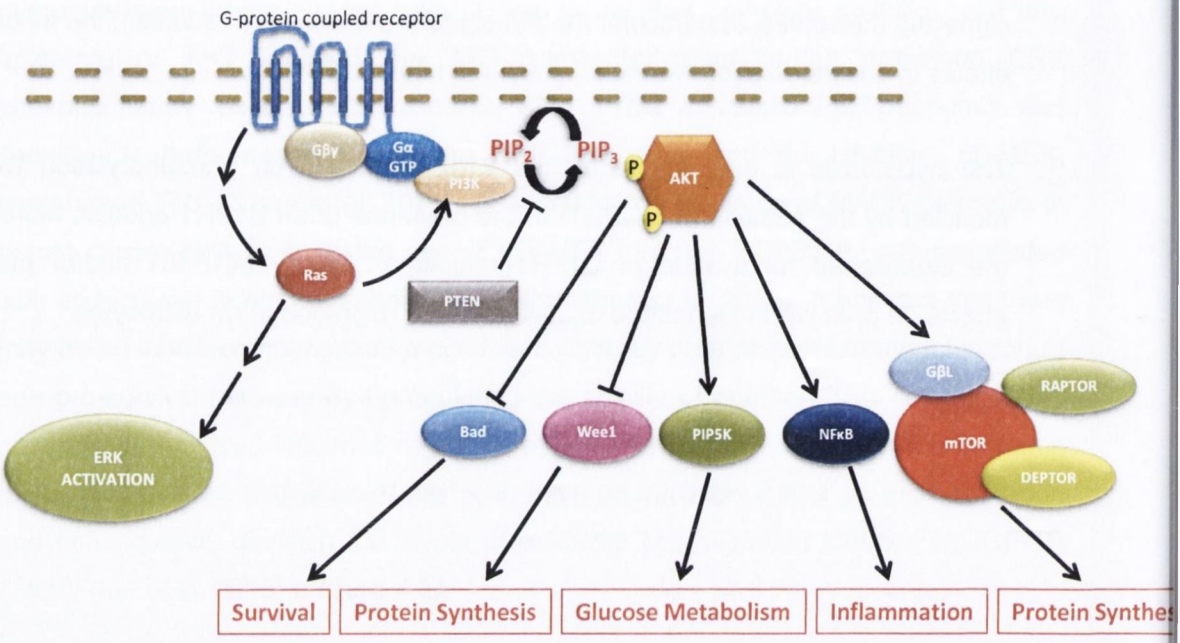

Figure 4.1 Mechanism of Akt phosphorylation and selected downstream substrates

PI3K can be activated by GPCRs (as shown) as well as growth factor receptors such as RTKs. PI3K overcomes inhibition by PTEN and converts PIP2 to PIP3, which in turn phosphorylates Akt. Phosphorylated Akt is a signalling molecule that activates numerous downstream molecules, while inhibiting the activation of others. In this manner, Akt regulates survival, protein synthesis, glucose metabolism and inflammation. It also shares an upstream activator (Ras) with another signalling molecules involved in cell survival, namely ERK.

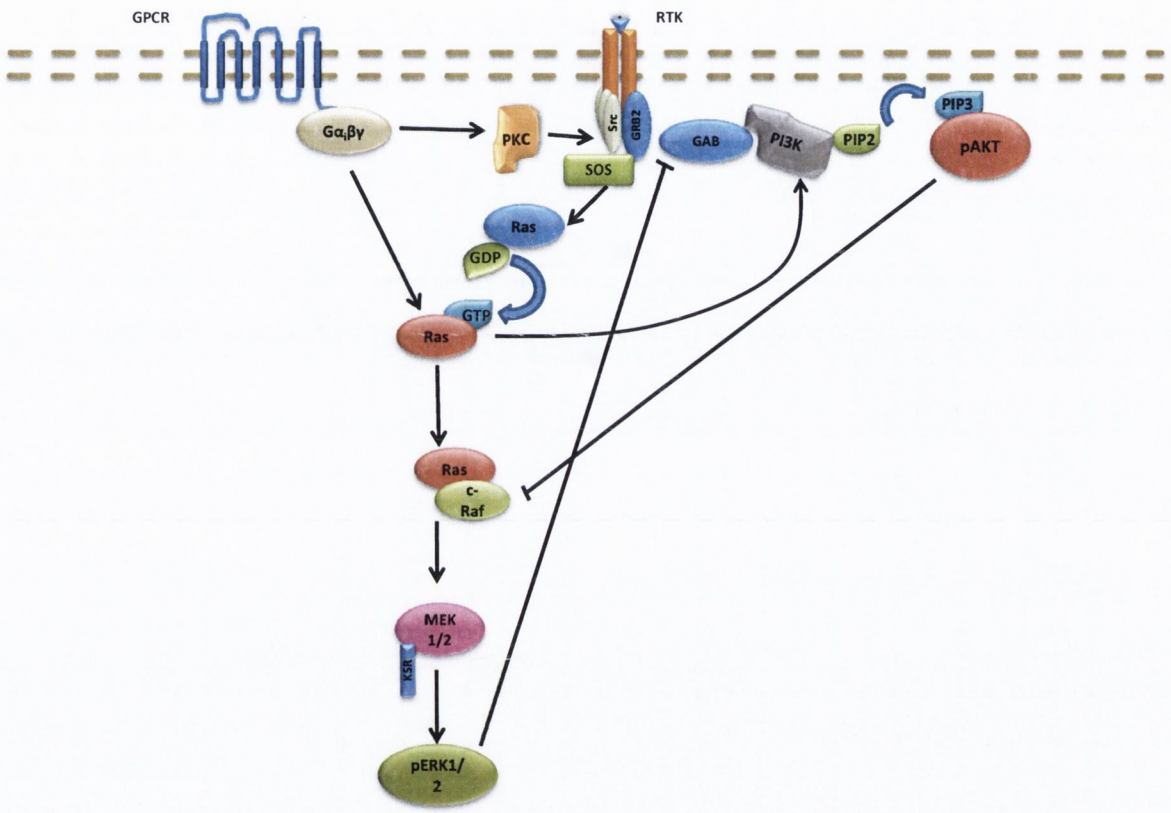

Figure 4.2 Scheme of cross-talk between ERK and AKT signalling pathways

The Akt and ERK pathways contain a common upstream activator in RAS. In addition, research has shown that the activation of one pathway may inhibit the activation of the other and vice-versa. G-protein recruitment may cross-activate RTKs via protein kinase C (PKC). RTKs and GPCRs both activate RAS which can activate the ERK and/or Akt pathways. Akt activation may inhibit C-Raf, upstream of ERK, while ERK activation may inhibit Grb2-associated binding protein (GAB), upstream of Akt (Mendoza et al. 2011).

2 Results

2.1 AUY954 induces moderate Akt phosphorylation in rat astrocytes in a time and concentration-dependent manner.

The relationship between the activation of S1PRs and the activation of the Akt signalling pathway has been described in various mammalian cell-types, particularly endothelial cells (Lee et al. 2001), fibroblasts (Baudhuin et al. 2004) and T cells (Liu et al. 2009). The initiation of this pathway by S1PR activation in astrocytes has not been examined. As Akt phosphorylation is a core event in the regulation of survival, proliferation and migration in numerous other cell types, the activation of this pathway via S1PR activation deserves attention. In this study both the time and concentration dependent phosphorylation of Akt upon activation of S1PR1 on rat astrocytes were examined. Using Western blotting it was demonstrated that the activation of the Akt pathway is transient, peaking between 10 and 30 minutes post-treatment. In addition, the effects are dose-dependent, with a maximal response seen at 100nM. This data indicates that the activation of S1PR1 by AUY954 induces the phosphorylation of Akt in the manner typical of GPCRs (**Figure 4.3**).

2.2 BYR attenuates S1PR1 induced Akt phosphorylation.

AUY954 is a selective agonist of S1PR1 as has been established both within this group (Healy et al., 2013) as well as externally (Pan et al., 2006). It potently induces calcium signalling and ERK phosphorylation in astrocytes (Healy et al, 2013) however the activation of Akt by this compound appears to be more moderate (**Figure 4.3**). In order to further probe the effect of S1PR1 activation on the initiation of Akt phosphorylation, the S1PR1 antagonist BYR185 was used in conjunction with AUY954 to ascertain whether it could fully inhibit the effects of the agonist. As would be expected, BYR185 treatment alone had no effect on the levels of pAkt when compared to control (114 ± 16.6% SEM of untreated control) but it attenuated the levels of pAkt induced by AUY treatment (182 ± 13.5% SEM of untreated control), returning them to levels comparable to control (105 ± 34.4% SEM) (**Figure 4.4**).

2.3 MNP301 enhances S1PR1 induced Akt phosphorylation.

As described in Healy et al (2013), the phosphorylation of ERK in rat astrocytes, induced through the activation of S1PR1, is restricted by the addition of MNP301. This peptide has been proposed to be a pathway specific modulator of S1PR1 signalling as, in addition to ERK phosphorylation; it has also been shown to down-regulate S1PR1 calcium signalling while leaving the inhibition of cAMP unaffected (Healy et al., 2013).

In this current study the effects of MNP301 on the activation of Akt were examined. Interestingly, rather than the inhibitory effect seen in relation to calcium and ERK signalling, the peptide was shown to potentiate the phosphorylation of Akt by AUY954 after 10 minutes (209 ± 9.3% SEM of untreated control)($p<0.05$ based on one-way ANOVA and Newman-Keuls post hoc test) when compared to treatment with AUY954 alone (170 ± 5.2% SEM of untreated control) (**Figure 4.5**). At a later time-point of 30minutes, co-treatment of MNP301 and AUY displayed an increased effect (268.7 ± 7.5% SEM of untreated control) ($p<0.01$ based on one-way ANOVA and Newman-Keuls post hoc test) whereas AUY954 in isolation was broadly the same (179.9 ± 10% SEM of untreated control) as at 10 minutes (**Figure 4.5**). Taken together these results indicate that MNP301 enhances and possibly prolongs the phosphorylation of Akt induced by AUY954. The underlying mechanism and functional effects of this augmentation warranted closer investigation.

2.4 Inhibition of MEK by U0126 induces an increase in Akt phosphorylation that is enhanced and prolonged by the addition of AUY.

As described previously, MNP301 inhibits ERK phosphorylation while enhancing Akt phosphorylation induced by AUY954. Interaction between these signalling pathways has been described as cross-talk, whereby reduction of activity in one pathway gives rise to increased activity in the other, possibly through the removal of a negative feed-back loop (Irie et al. 2005; Serra et al. 2011; Normanno et al. 2006; Mendoza et al. 2011). In this study, it was noted that the S1PR1 activation of the ERK and Akt pathways occurred on broadly similar time-scales and the effects of MNP301 on Akt phosphorylation could be due to an inhibition of the ERK phosphorylation pathway. In order to explore this possibility, U0126 was used as an inhibitor of MEK, a direct upstream activator of ERK, and thus is a potent inhibitor of ERK phosphorylation. It was found that U0126 induced time-dependent activation of the Akt pathway and further enhanced and prolonged the activation of Akt induced by AUY954 treatment. It was seen that there was a significant difference between AUY954 treatment and AUY954 and U0126 co-treatment at 60 (98 ± 10.1% vs. 214.1 ± 18.2% SEM of control) ($p<0.01$ based on student T-test comparing means of time-matched samples) and 90 minutes (112.4 ± 3.3% vs. 243 ± 31.7% SEM of control) ($p<0.01$ based on student T-test comparing means of time-matched samples) (**Figure 4.6**). Furthermore, when U0126 mono-treatment was compared to U0126 and AUY954 co-treatment there was a significant difference between treatments at 30 minutes (128.1 ± 5.9 % vs. 193.7 ± 23.7% SEM of control) (**Figure 4.6**). Taken together, these data show that the

inhibition of the phosphorylation of ERK can indeed bring about an increased phosphorylation of Akt, likely through the removal of a negative feedback mechanism regulated by pERK. Further, this provides a plausible explanation as to why MNP301 inhibits ERK phosphorylation while increasing Akt phosphorylation. The functional outcomes of this intracellular signalling modulation were next to be examined by assessing the effects of cross-talk on astrocyte migration.

2.5 Promotion of astrocyte migration by pFTY720 and AUY954 is dose dependent.

Mullershausen et al. (2007) demonstrated that the activation of S1PRs by pFTY720 could induce the migration of rat astrocytes using a scratch-wound assay. It was further proposed that this effect was regulated through S1PR1 as the effects of an S1PR1-specific agonist were comparable to those seen with pFTY720 treatment. In order to validate this research and establish an assay through which the functional effects of modulation of S1PR1 signalling could be examined, a novel migration assay was developed. Strips of silicon, 0.5mm wide, were used to prevent astrocyte growth on a coverslip. In this way, it was possible to mimic the scratch-wound and create an astrocyte-free gap of consistent width while also limiting cell damage that might be incurred by creating a wound. Using this assay technique it was demonstrated that both pFTY720 and AUY954 induce astrocyte migration significantly greater that control in a dose-dependent manner (**Figure 4.7**). Both agonists displayed effects that matched a bell-shaped curved, a common effect when examining chemotaxis (Campbell et al., 1996, Wain et al., 2002 & Tchernychev et al. 2010). A maximal response was seen with 10nM pFTY720 (478.7 ± 117% SEM of control) ($p<0.01$ based on one-way ANOVA and Newman-Keuls post hoc test) and 100nM AUY954 (532.7 ± 172% SEM of control) ($p<0.05$ based on one-way ANOVA and Newman-Keuls post hoc test) at 24 hours post-treatment (**Figure 4.7**).

2.6 Astrocyte migration induced by AUY954 and pFTY720 is ablated by BYR185.

AUY954 is a specific agonist of S1PR1, while pFTY720 has the ability to activate S1PR1 as well as S1PR3 and S1PR5s that are expressed on astrocytes. In order to ascertain if there is an additional signalling component beyond activation on S1PR1 in the promotion of astrocyte migration, the S1PR1-sepecific antagonist BYR185 was utilised. It was found that addition of BYR185 (1μM) 1 hour prior to treatment with either AUY954 (100nM) (14.9 ± 7.1% SEM of control) or pFTY720 (10nM) (6.5 ±

15.1% SEM of control) potently inhibited astrocyte migration induced by these compounds at 24 hours (**Figure 4.8**). Interestingly, the antagonist BYR185 reduced migration levels to below that of control (23.27 ± 22% SEM of control migration) indicating that S1PR1 signalling is involved in astrocyte migration in the absence of exogenous agonists. This data supports the hypothesis that S1PR-associated astrocyte migration is primarily, if not solely, mediated through the G_i-coupled S1PR1.

2.7 Inhibition of Akt phosphorylation attenuates S1PR induced astrocyte migration.

In order to isolate the signalling mechanism that underlies the promotion of astrocyte migration induced by S1PR1 activation, an inhibitor of Akt phosphorylation was used. MK2206 is an inhibitor of Akt phosphorylation currently in clinical trials as an anti-cancer therapy (Yap et al. 2011). It is a specific inhibitor of Akt as opposed to an inhibitor of an upstream target, such as PI3K, and thus is less likely to interfere with other signalling pathways that may rely on PI3K. When examined in this migration assay it was seen that MK2206 was a strong inhibitor of chemotaxis (**Figure 4.9**). Astrocytes, pre-treated with MK2206 prior to stimulation with AUY954 showed no migration into the strip-area (70.1 ± 20.6% SEM of untreated control migration), much like the levels of movement seen in the MK2206+pFTY720 treated samples (55.7 ± 14.5% SEM of untreated control) in comparison to 3-5 fold of control distances migrated in the AUY954 and pFTY720 treated controls (**Figure 4.9**). These data suggest that Akt phosphorylation plays an important role in S1PR1 induced migration of rat astrocytes, a mechanism that has, as yet, been little described.

2.8 Inhibition of MEK/ERK phosphorylation promotes astrocyte migration.

The identification of Akt as a putative signalling molecule involved in the regulation of S1PR1 induced astrocyte migration does not remove the possibility that other signalling molecules may play a similar role. To further clarify the mechanisms involved in this astrocyte migration, the role of the ERK pathway was examined. U0126 is an inhibitor of MEK activation and as MEK is a direct upstream activator of ERK, the inhibition of this kinase prevents the phosphorylation of ERK. This inhibition of MEK/ERK has already been shown to promote the concomitant activation of Akt in astrocytes in this study (**Figure 4.6 C**). However the functional outcomes of this phenomenon have not been explored. It was found that U0126 (1μM) promoted astrocyte migration to levels similar to that seen in AUY954 and pFTY720 treated samples (353.3 ± 90.1% SEM of untreated control) and that co-treatment with either

AUY954 or pFTY720 did not significantly alter the pattern of migration when compared to mono-treatment (402.2 ± 63.7% and 305.2 ± 32.1% SEM of untreated control respectively) (**Figure 4.10**). Based on these data using U0126, in conjunction with the results described in experiments utilising MK2202, it appears that restricting the phosphorylation of ERK may induce activation of the Akt pathway, thereby increasing levels of astrocyte migration. Also, due to the efficacy of U0126 in promoting migration in the absence of any specific receptor activation, there are grounds to conclude that there is a constitutive inhibition exerted on the Akt pathway by the ERK pathway. Furthermore, removal of this inhibition by inhibition of basal ERK phosphorylation is enough to promote Akt phosphorylation and migration of astrocytes.

2.9 MNP301 promotes astrocyte migration independent of S1PRs.

MNP301 has been shown to induce S1PR1 specific and pathway selective modulation of intracellular signalling in astrocytes. In addition to published data indicating that MNP301 exerts an effect on calcium and ERK signalling (Healy et al. 2013), the data in this chapter shows that MNP301 augments the phosphorylation of Akt induced by AUY954 (**Figure 4.3**). In order to investigate the functional consequences of the peptide's effects on intracellular signalling, astrocytes in this migration assay were treated with MNP301 (100µg/ml) in the presence or absence of AUY954 (**Figure 4.11**). It was seen that MNP301 induced a significant level of migration, in the presence of AUY954 (538.2 ± 52.25% SEM of untreated control) as well as in the absence of AUY954 (603.2 ± 71.33% SEM of untreated control) These levels of migration were similar to those seen in samples treated with AUY954 alone (451.8 ± 42.8% SEM of untreated control) ($p<0.001$ based on one-way ANOVA and Newman-Keuls post-hoc test) (**Figure 4.11**). Similarly, when MNP301 was examined in the presence or absence of pFTY720 there was little difference between the levels of migration seen with pFTY720 (360.2 ± 90% SEM of untreated control) peptide alone (307.8 ± 20.1% SEM) and the peptide in the presence of pFTY (346.9 ± 19.9% SEM of untreated control) (**Figure 4.11**). The ability of MNP301 to induce such high levels of migration was somewhat unexpected and could be attributed to a potentiation of Akt phosphorylation as described previously.

2.10 MK2206 reduces astrocyte migration induced by MNP301 and U0126.

The data as described so far indicates that there is crosstalk between the ERK and Akt pathways that, under altered equilibrium, regulates astrocyte migration. Inhibition of ERK activity, even without S1PR1 activation, promotes Akt phosphorylation and this

leads to the production of a migratory signal for astrocytes. Confirmation of this hypothesis requires validation that Akt phosphorylation is necessary for the migratory effects seen upon treatment of astrocytes with MNP301 and U0126. In order to acquire this confirmation, MK2206 (1μM) was used to inhibit Akt activation in the presence of either MNP301 (100μg/ml) or U0126 (1μM). In line with the hypothesis expressed, MK2206 reduced the levels of migration induced by both treatments. MNP301 induced a mean 463.5% (±54.7% SEM) of control migration whereas samples treated with both MNP301 and MK2206 actually retreated on average (-19.6 ± 25.13% SEM) (**Figure 4.12**). The effects seen with U0126 were less striking but still equated to a halving of the migratory distance (461.5 ±81.1% vs. 230.6 ± 70.1% SEM of untreated control) (**Figure 4.12**). Overall these data strongly support the hypothesis that both MNP301 and U0126 promote astrocyte migration through the removal of the inhibitory feedback that the ERK pathway constitutively exerts over the Akt pathway. Akt phosphorylation is therefore proposed as an integral part of the mechanism regulating not only S1PR1-associated astrocyte migration but also astrocyte migration in general.

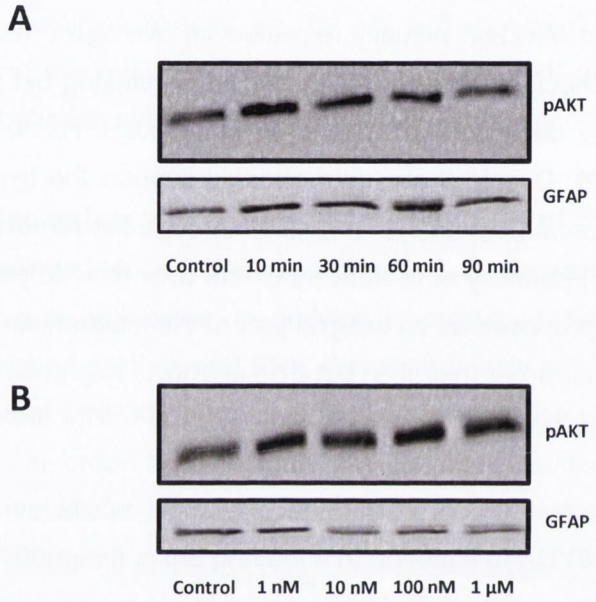

Figure 4.3 AUY954 induces moderate Akt phosphorylation in a time and concentration-dependent manner.

(A) Rat astrocytes were serum starved for 3hr and treated with 10nM AUY954 for 5-60 min, after which cells were scraped and processed for Western blotting. **(B)** Cells were serum starved for 3hr and treated with1nM-1µM AUY954 for 10 min, after which cells were scraped and processed for Western blotting. (N=4 independent experiments)

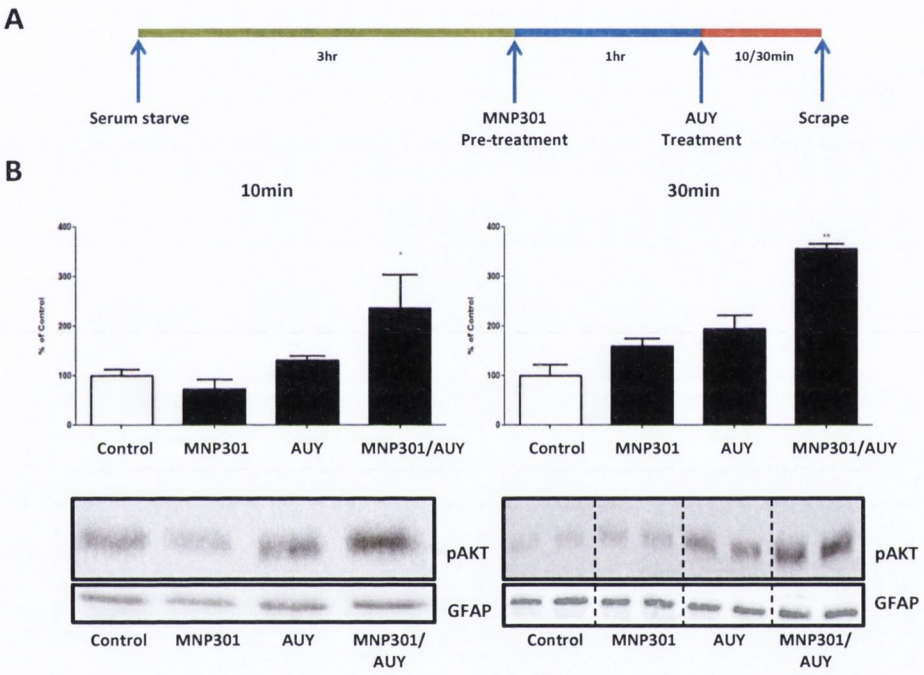

Figure 4.4 MNP301 enhances S1PR1 induced AKT phosphorylation

(A) Rat astrocytes were serum starved for 3h, pre-incubated with 100µg/ml MNP301 for 1h and then treated with 10nM AUY954 for 10 or 30 min, after which cells were scraped and processed for Western blotting. **(B)** Treatment of cells with MNP301 and AUY induces AKT phosphorylation that is greater than phosphorylation induced by either compound in isolation. *p<0.05, **p<0.01 significantly different to DMSO control based on one-way ANOVA and Newman-Keuls post-hoc test. Graphs depict mean ± SEM. (N=4 independent experiments)

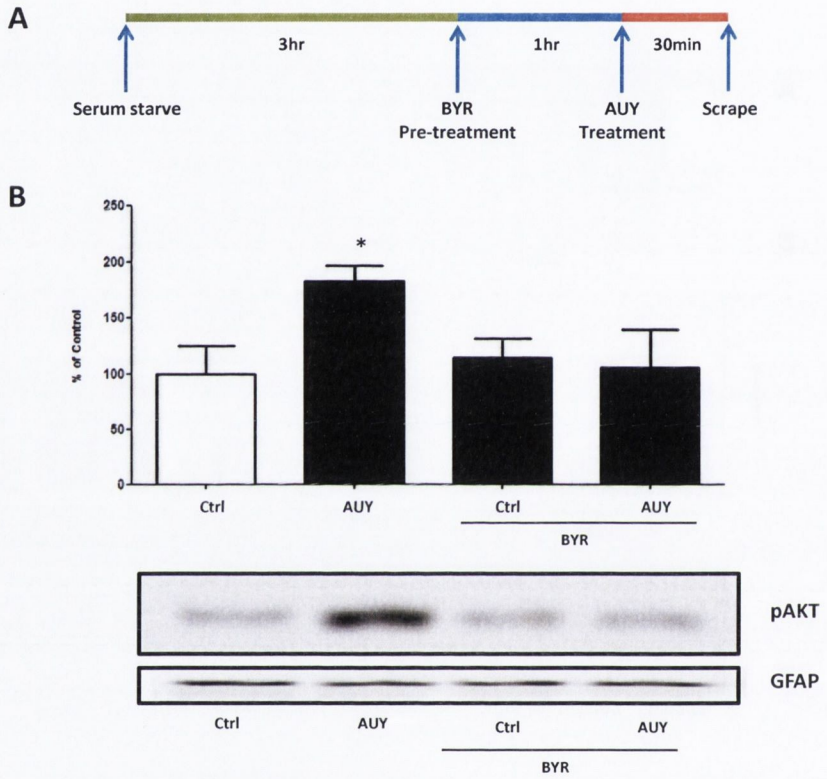

Figure 4.5 BYR attenuates S1PR1 induced pAkt

(A) Rat astrocytes were serum starved for 3h, pre-incubated with BYR185 (1μM) for 1h and then treated with AUY954 (10nM) for 30 min, after which cells were scraped and processed for Western blotting. **(B)** BYR185 attenuates AUY954 induced Akt phosphorylation. *$p<0.05$, significantly different to DMSO control based on one-way ANOVA and Newman-Keuls post-hoc test. Graph depicts mean ± SEM (N=4 independent experiments)

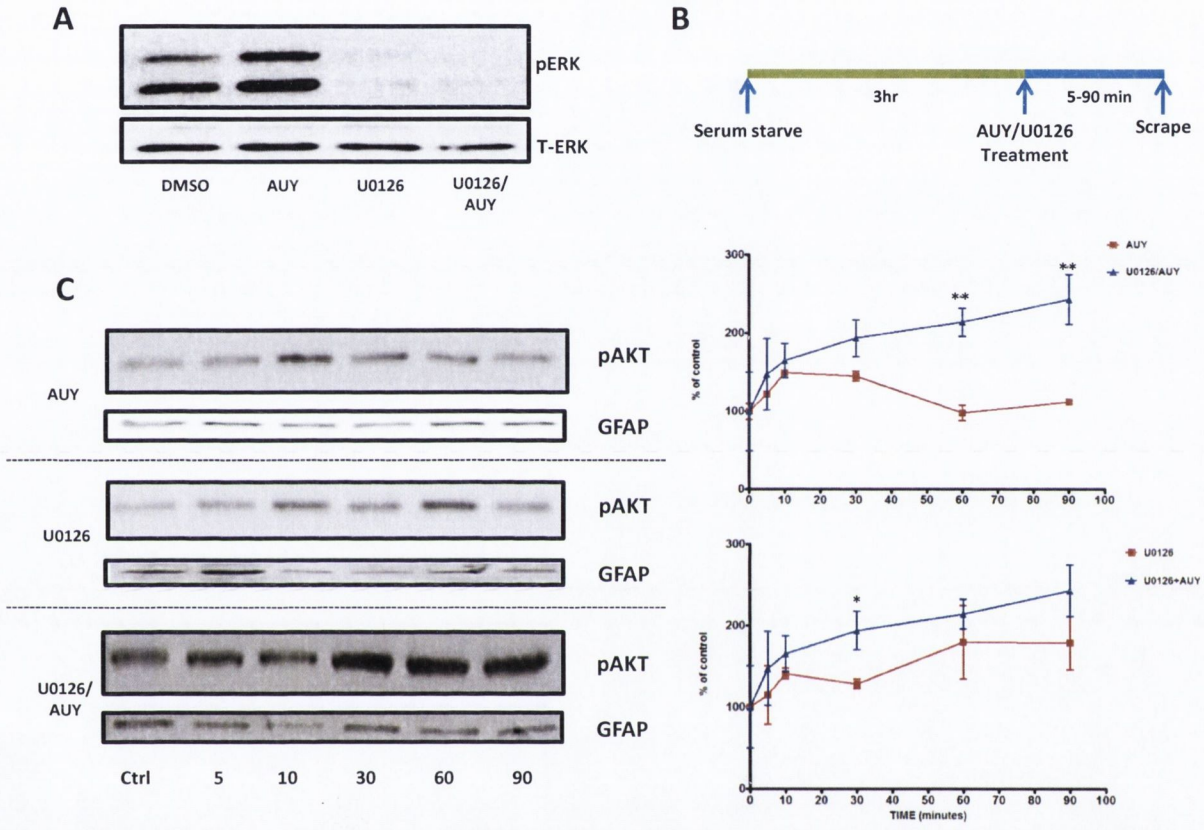

Figure 4.6 Inhibition of MEK by U0126 induces an increase in AKT phosphorylation that is enhanced and prolonged by the addition of AUY.

(A) The MEK inhibitor, U0126 (25μM), potently inhibits ERK phosphorylation by AUY954 (10nM). **(B)** Rat astrocytes were serum starved for 3h, incubated with AUY954 (10nM) and/or U0126 (25μM) for 5-90min, after which cells were scraped and processed for Western blotting. **(C)** Co-treatment of AUY954 with U0126 induced enhanced and prolonged phosphorylation of pAkt when compared to AUY treatment or U0126 alone. *p<0.05, **p<0.01 significantly different to time-matched treatment based on Student's t-test. Graphs depict mean ± SEM (N=3 independent experiments)

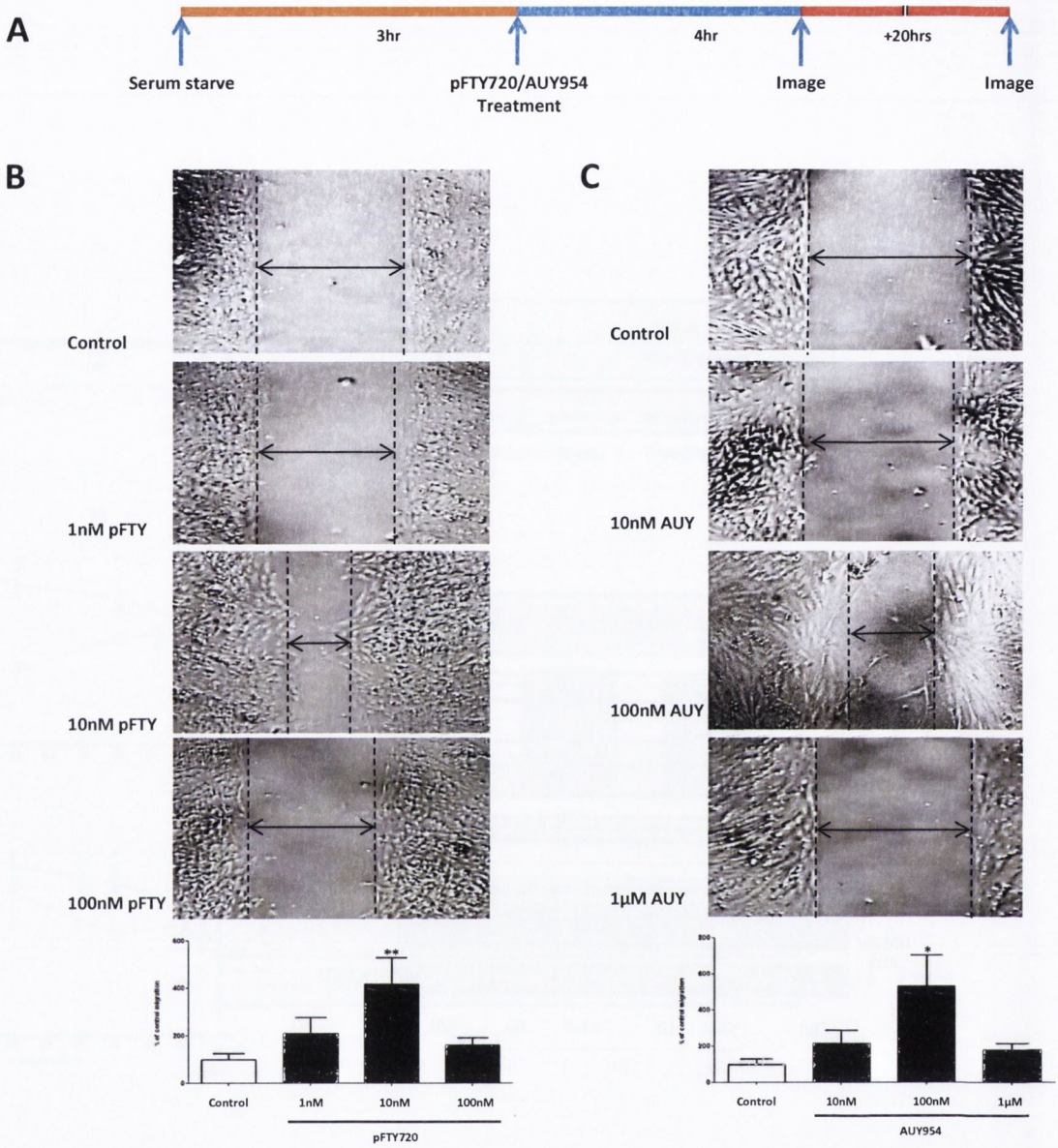

Figure 4.7 Promotion of astrocyte migration by pFTY720 and AUY954 is concentration-dependent.

(A) Rat astrocytes were serum starved for 3h before being treated with 1-100nM pFTY720 or 10-1000nM AUY954. Images were captured 4hrs and 24hrs post-treatment. **(B)** Representative images display concentration dependent migration of astrocytes induced by pFTY720. Graph displays the mean distance between (+/-SEM) migration fronts at 4hrs and 24hrs post-treatment. **(C)** Representative images display concentration dependent migration of astrocytes induced by AUY954. Graph displays the mean distance (+/-SEM) between migration fronts at 4hrs and 24hrs post-treatment. *p<0.05, p<0.01 significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. (N=3 independent experiments)

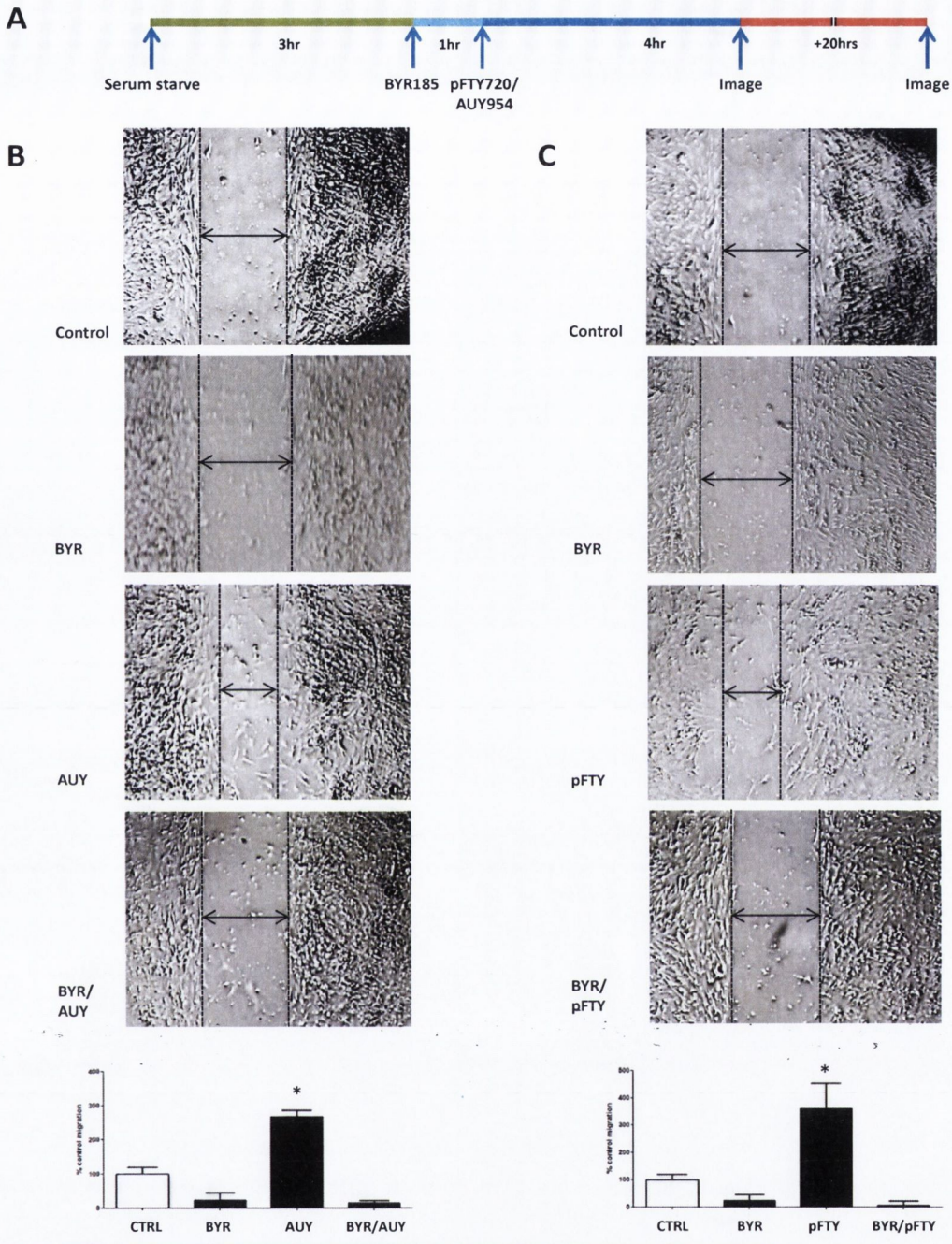

Figure 4.8 Astrocyte migration induced by AUY954 and pFTY720 is ablated by BYR185. **(A)** Cells were serum starved for 3h before being treated with 100µg/ml MNP301, after a further hour pFTY720 (10nM) or AUY954 (100nM) was added. Images were captured 4hrs and 24hrs post-treatment. **(B)** Representative images display AUY induced migration of astrocytes is attenuated by BYR. Graph displays the mean (+/-SEM) distance between migration fronts at 4hrs and 24hrs post-treatment. **(C)** Representative images display pFTY720 induced migration of astrocytes is attenuated by BYR. Graph displays the mean (+/-SEM) distance between migration fronts at 4hrs and 24hrs post-treatment *$p<0.05$ significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. (N=3 independent experiments)

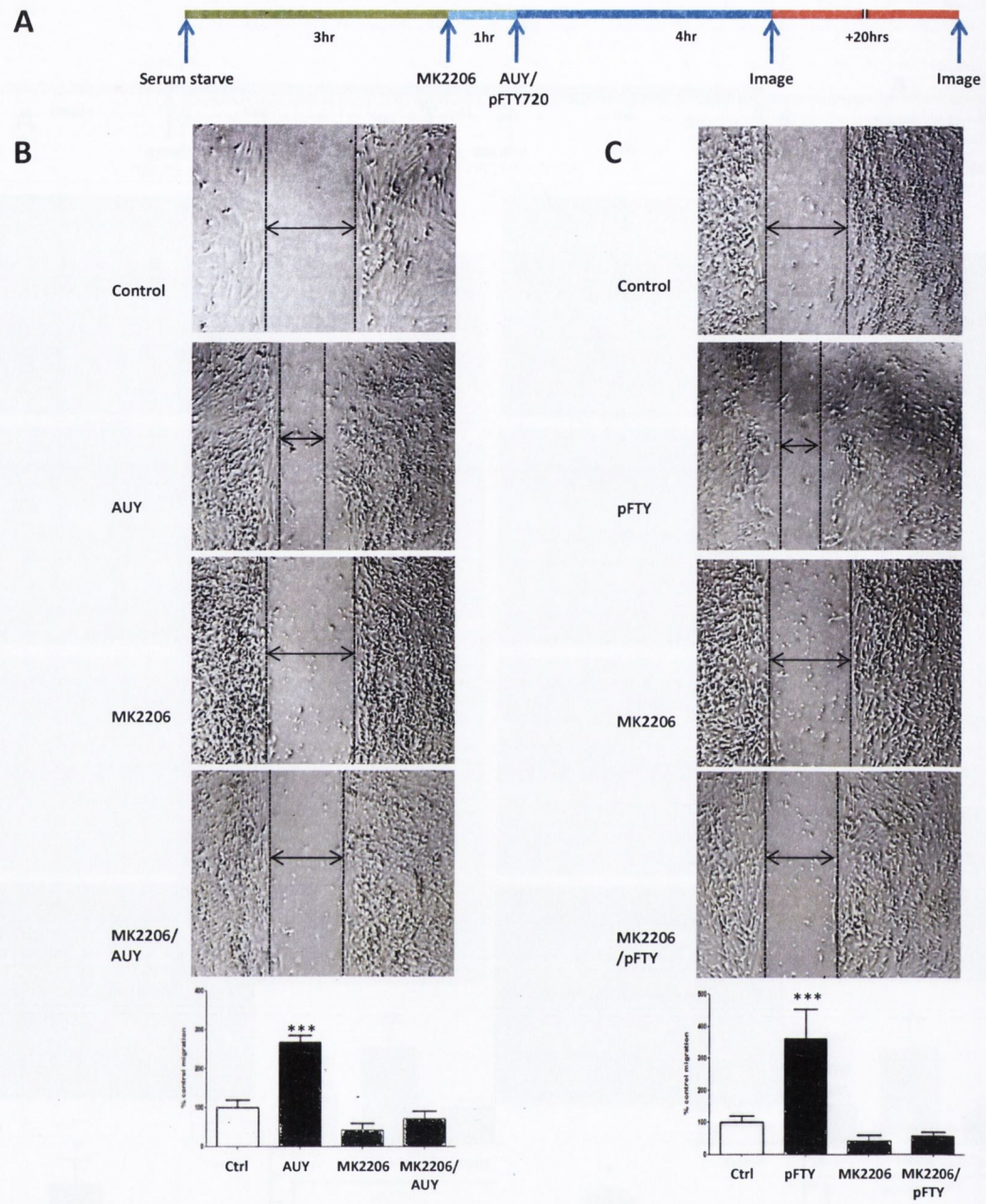

Figure 4.9 Inhibition of AKT phosphorylation attenuates S1PR1 induced astrocyte migration.

(A) Astrocytes were serum starved for 3h before being treated with 1μM MK2206 for 1h and then treated with pFTY720 (10nM) or AUY954(100nM). Images were captured 4hrs and 24hrs post-treatment. **(B)** Representative images display relative migration of astrocytes. Graph displays the mean (+/-SEM) distance between migration fronts at 4hrs and 24hrs post-treatment. MK2206 (1μM) attenuates astrocyte migration induced by AUY954 **(C)** Representative images display relative migration of astrocytes. Graph displays the mean (+/-SEM) distance between migration fronts at 4hrs and 24hrs post-treatment. MK2206 (1μM) attenuates astrocyte migration induced by pFTY720 ***$p<0.01$ significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. (N=5 independent experiments)

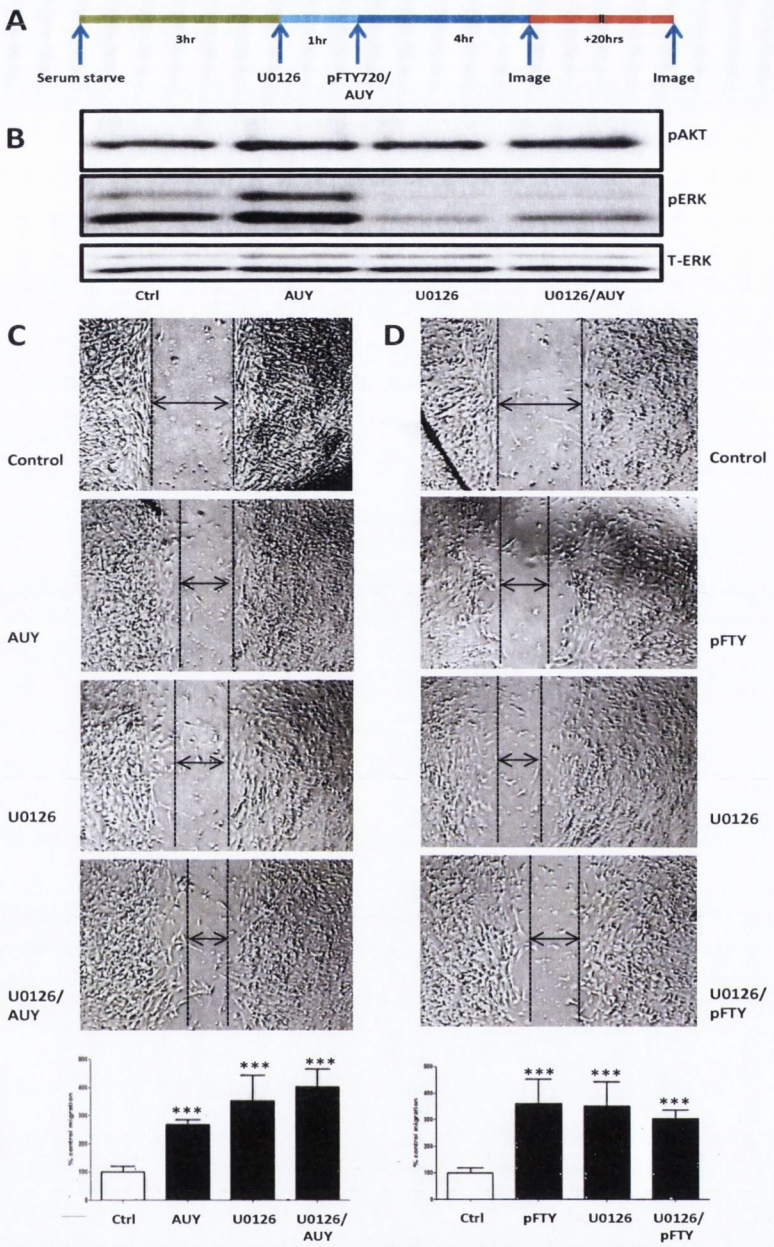

Figure 4.10 U0126 promotes astrocyte migration independent of S1PR1 activation

(A) Astrocytes were serum starved for 3h before being treated with 1μM U0126 for 1h and then treated with 100nM AUY954 or 10nM pFTY720. Images were captured 4hrs and 24hrs post-treatment. **(B)** At 1μM concentration U0126 inhibits ERK phosphorylation induced by AUY (10min at 10nM) and induces activation of AKT **(C)** Representative images display relative migration of astrocytes. Graph displays the mean (+/- SEM) distance between migration fronts at 4hrs and 24hrs post-treatment.U0126 promotes astrocyte migration independent of AUY954. **(D)** Representative images display relative migration of astrocytes. Graph displays the mean (+/- SEM) distance between migration fronts at 4hrs and 24hrs post-treatment.U0126 promotes astrocyte migration independent of pFTY720. ***$p<0.01$ significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. (N=3 independent experiments)

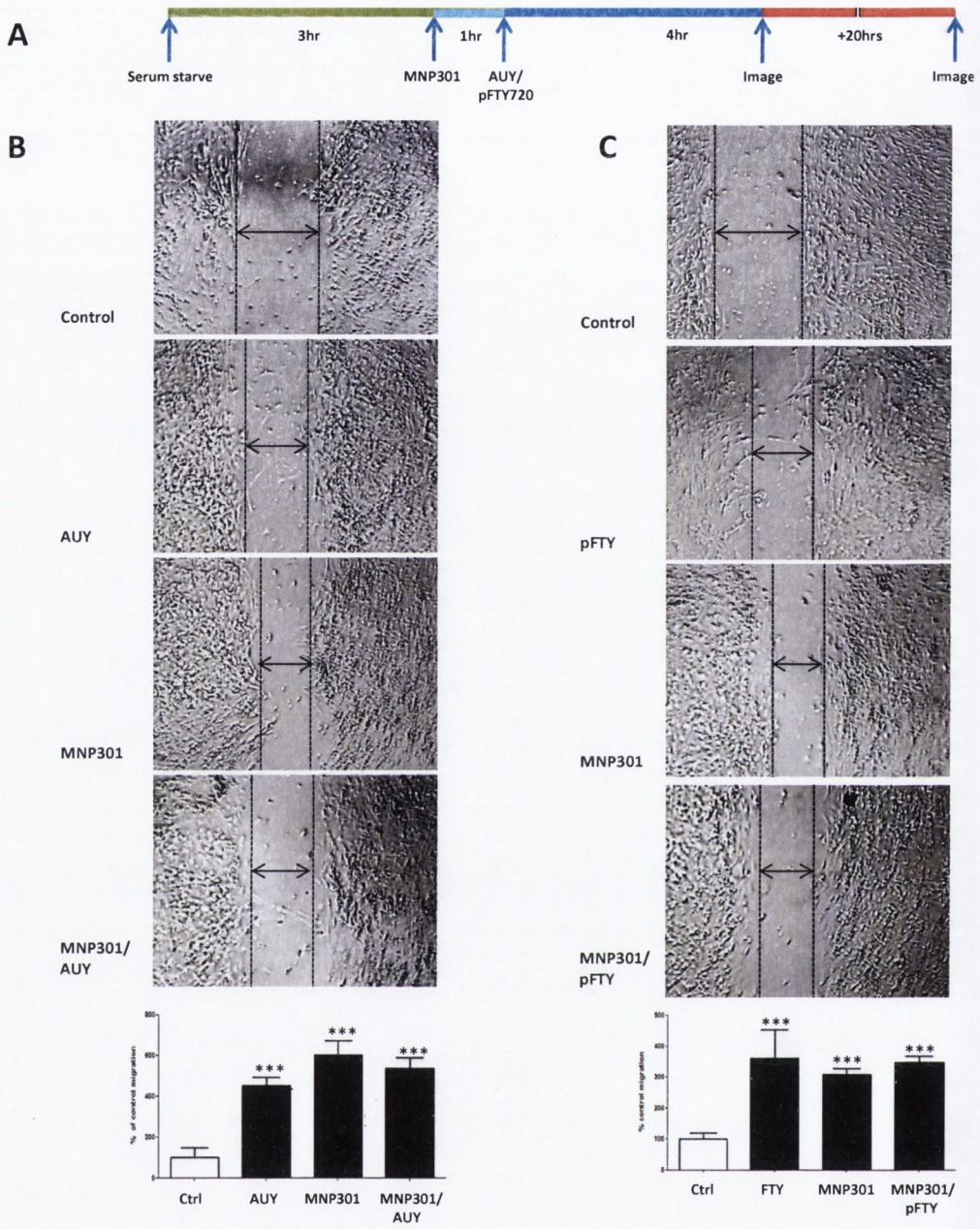

Figure 4.11 MNP301 promotes astrocyte migration independent of S1PR1 activation

(A) Astrocytes were serum starved for 3h before being treated with 100µg/ml MNP301, after a further hour AUY954 (100nM) or pFTY720 (10nM) were added. Images were captured 4hrs and 24hrs post-treatment. **(B)** Representative images display MNP301 +/-AUY954 induced migration of astrocytes. Graph displays the mean distance (+/-SEM) between migration fronts at 4hrs and 24hrs post-treatment. MNP301 and AUY954 promote astrocyte migration to a similar extent. **(C)** Representative images display MNP301 +/- pFTY720 induced migration of astrocytes. Graph displays the mean (+/-SEM) distance between migration fronts at 4hrs and 24hrs post-treatment. MNP301 and pFTY720 promote astrocyte migration to a similar extent. ***p<0.001, significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. (N=5 independent experiments)

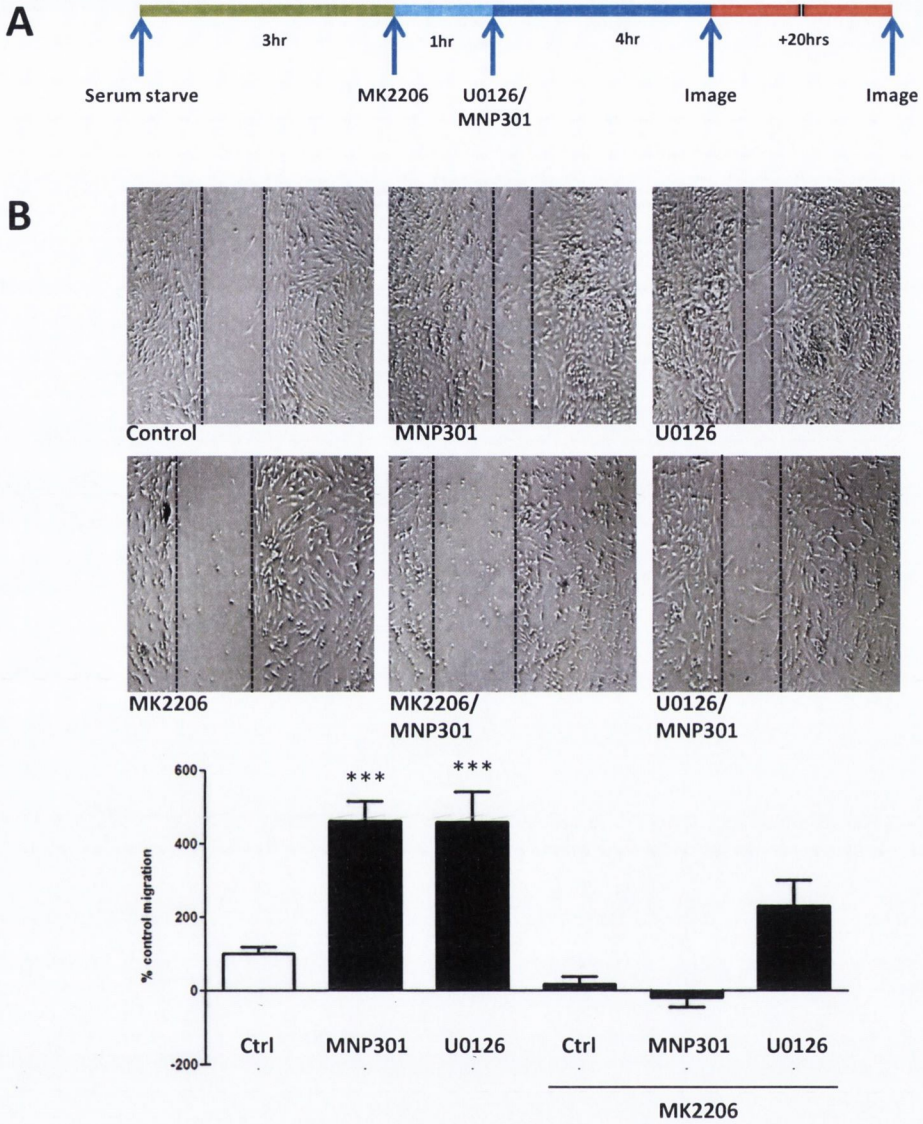

Figure 4.12 MK2206 reduces astrocyte migration induced by MNP301 and U0126.

(A) Astrocytes were serum starved for 3h before being treated with MK2206 (1μM) for 1 hour followed by U0126 (1μM) or MNP301 (100μg/ml). Images were captured 4hrs and 24hrs post-treatment. **(B)** Both U0126 and MNP301 promote significant levels of astrocyte migration which is ablated by the addition of MK2206. ***$p<0.01$ significantly different to untreated control based on one-way ANOVA and Newman-Keuls post-hoc test. (N=3 independent experiments)

3 Discussion

3.1 Do S1PRs regulate Akt phosphorylation in rat astrocytes?

Activation of S1PR1 causes a moderate and transient phosphorylation of Akt. A link between S1PR1 activation and Akt phosphorylation has been noted in many cell types but has not been described extensively with regard to any cell type in the CNS. In this study it was found that the activation of S1PR1 by the specific agonist AUY954 induced a moderate but consistent increase in the levels of pAkt that was both time and concentration dependent. Maximal activation occurred at approximately 100nM and between 10 and 30 minutes post-treatment. In addition, this activation was fully reversed by the addition of BYR185, further confirming the ability of S1PR1 activation to induce phosphorylation of Akt. These results were broadly similar to those seen when the phosphorylation of ERK, via S1PR1 activation, was examined. This is an unsurprising observation as many reports have described close links between the Akt and ERK pathways, not only in functional outcomes of activation but also in regard to cross-talk (Irie et al. 2005; Serra et al. 2011; Normanno et al. 2006; Mendoza et al. 2011) and over-lap of upstream activators, such as Ras (Chappell et al. 2011). These two separate pathways are prime novel targets for the treatment of cancers due to their shared importance in cellular growth and proliferation (Chappell et al. 2011; De Luca et al. 2012). Once it was established in this study that AUY954-induced activation of pERK was inhibited by the use of the pathway specific modulator, MNP301 (Healy et al. 2013) and that the same agonist also induced an increase, albeit moderate, in the levels of pAkt, the effects of the MNP301 peptide were investigated in the Akt signalling pathway. The commonality of upstream activators of pERK and pAkt lead to a proposed hypothesis that MNP301 may inhibit pAkt in a manner similar to that seen with pERK.

3.2 Does MNP301 modulate S1PR1 induced pAkt?

MNP301 is a peptide that mimics the final ten amino acids of the S1PR1 and is tagged with a TAT peptide in order to allow it to cross the plasma membrane. The ability of the peptide to permeate the cell was demonstrated by Healy et al. (2013) in both astrocyte cell culture and in organotypic brain slices. It was further shown that this peptide reduced levels of calcium signalling and the phosphorylation of ERK induced through S1PR1 **(Chapter 3)** specifically but had no effect on the reduction of cAMP (Healy et al. 2013). Due to the described effects of MNP301 on the pathways mentioned, S1PR1 induced Akt phosphorylation was also examined. Contrary to expectations, MNP301 in conjunction with AUY954 promoted the phosphorylation of Akt beyond that seen with

AUY954 alone. The difference in Akt phosphorylation between MNP301 and AUY954 co-treatment and AUY954 alone was also accentuated over time, with a greater divergence seen at 30 minutes than at 10 minutes. This disparity was viewed as evidence that MNP301 may prolong the effect of AUY954 induced Akt phosphorylation as that had previously been shown to be transient.

The mechanism through which MNP301 could potentiate AUY954-induced Akt phosphorylation was unclear but it was hypothesized that cross-talk between the ERK pathway and Akt pathway may play a role. In order to investigate this possibility, the MEK inhibitor U0126 was used to prevent ERK phosphorylation in the presence of AUY954 activation of S1PR1. In these experiments it was demonstrated that the addition of U0126 induced activation of Akt to a similar level as that seen with AUY954. This indicated that reduction of basal ERK phosphorylation, in the absence of any specific cell-surface receptor agonism, was enough to induce activation of Akt. Furthermore, co-treatment of astrocytes with both AUY954 and U0126 initiated a synergistic or additive effect that was similar to that seen when astrocytes were co-treated with AUY954 and MNP301. In these experiments co-treatment with U0126 and AUY954 significantly enhanced and prolonged the phosphorylation of Akt when compared to either treatment alone. This effect of U0126 demonstrated that the putative mechanism by which MNP301 promoted AUY954 induced pAkt was due to an inhibition of pERK and the removal of inhibitory feedback on the Akt pathway. This is not an unknown mechanism and enhancement of one of these pathways in conjunction with inhibition of the other has been described in respect to epithelial cells (Irie et al. 2005) and in cancer cells from breast tissue (Normanno et al. 2006; Serra et al. 2011).

3.3 What are the functional outcomes of the enhancement of S1PR1-induced pAkt signalling?

Mullershausen et al (2007) demonstrated that activation of S1PRs promotes astrocyte migration in a scratch-wound assay system. Due to the similar efficacy of both pFTY720 and SEW2871, these effects were deemed to be S1PR1-mediated. A version of this assay, in which the scratch-wound was replaced by a silicon strip that prevented cell growth along a narrow 0.5mm wide corridor, was developed in order to examine the functional consequences of the regulation of S1PR1-stimulated pAkt and pERK. The silicon strip was utilized in order to reduce the possibility of cell damage caused by the scratch-wound and also to create a corridor of consistent width across

the entire cover-slip. Using this assay system it was established that both pFTY720 (10nM) and AUY954 (100nM) caused significant migration of rat astrocytes across the cell-free area created by the silicon strip when compared to control, in accordance with the results described by Mullershausen et al. (2007). The S1PR-induced migration of astrocytes was fully inhibited by the addition of the S1PR antagonist BYR185, thus indicating that S1PR1 is the receptor responsible for both AUY954 and pFTY720 associated migration. Interestingly, BYR185 also reduced the level of migration below that seen in control samples thus indicating that the S1PR1 is actively involved in the regulation of migration in the absence of the introduction of an exogenous agonist. This may be accounted for by S1P release by astrocytes *in vitro* acting in an autocrine or paracrine manner on the S1PR1.

The pathways involved in the regulation of this cellular response to receptor activation were still unknown; therefore, MK2206 was used to establish whether pAkt was a component of this signalling mechanism. Treatment of astrocytes with this inhibitor of Akt attenuated the migration caused by AUY954 and pFTY720 indicating that the phosphorylation of Akt was essential for S1PR1-generated migration. This data falls into line with a previous study that showed that S1PR1 activation may influence migration of endothelial cells via an Akt-dependent mechanism (Lee et al. 2001). This cited study proposed a mechanism by which Akt regulated S1PR1 phosphorylation post-activation and independent of G_i-signalling however a similar mechanism was not examined in the current study.

After it was established that pAkt was an integral part of the signalling cascade involved in the control of S1PR1-related astrocyte migration, the effect of MEK/ERK inhibition and the associated increase in pAkt was explored. In contrast to MK2206, the MEK inhibitor U0126 did not impair the ability of AUY954 or pFTY720 to initiate astrocyte migration. Rather, this compound promoted significant migration by itself, independent of S1PR activation. Based on these data, it was proposed that the inhibition of ERK by U0126 was a potential mechanism through which an increase in phosphorylation of Akt was achieved, thus mimicking the effects of S1PR1 activation of the same pathway. This would explain the similar levels of migration seen when comparing U0126 treated cells with those treated with either AUY954 or pFTY720. There did not appear to be any additive effect on astrocyte migration when either of the S1PR agonists was used in conjunction with U0126 as had been seen when AUY954 and U0126 were used to acutely induce a synergistic phosphorylation of Akt. However, the equivalence in the levels of migration seen between individual and co-

treatments may have been due to the migration response reaching a maximal threshold, as all agonist and inhibitors remained present in the media throughout the 24hour time-course.

In light of these findings it remained to be established whether MNP301 exerted effects on the migration of astrocytes comparable to those seen with U0126. MNP301 promoted migration with or without the addition of a S1PR agonist, in agreement with the effect on migration induced by U0126. Furthermore, there was no additive effect seen when MNP301 was co-applied with either S1PR agonist, although as proposed previously this may be due to a maximal response being achieved. As with U0126, the mechanism through which MNP301 promotes astrocyte migration is suggested to relate to regulation of the cross-talk between the ERK and Akt pathways. To confirm a role for Akt phosphorylation in the U0126/MNP301-promotion of astrocyte migration, the effect of the Akt inhibitor MK2206 was investigated. MK2206 completely ablated the migratory effect of MNP301 while diminishing the effects seen with U0126 treatment. The fact that MK2206 did not attenuate U0126 induced migration to the same degree as it did with MNP301 is likely due to the fact that equal concentrations MK2206 and U0126 were used. Based on biochemical kinase assays MK2206 has an IC50 of 8-65nM depending on the isoform of Akt (Yan 2009), whereas U0126 has an IC50 of 72/58nM depending on the MEK isoform in question (Favata 1998). It is proposed that a higher concentration of MK2206 relative to U0126 may have brought about a more potent inhibition of migration. Research has shown that there may be constitutive activity of S1PR1 when expressed in HEK cells (Waters et al. 2006). While Waters et al (2006) described that constitutive S1PR1 activation in HEK cells resulted in ERK activation via the PDGF receptor, constitutive S1PR1 activation could account for some of the basal level of ERK phosphorylation seen in astrocytes. Removal of this ERK signal by MNP301 could explain why the peptide induces astrocyte migration in an Akt dependent manner.

3.4 Conclusions

Overall, this chapter describes how the inhibition of one intracellular pathway, namely the ERK pathway, impacts on linked intracellular systems, namely Akt. Both ERK and Akt perform some similar functions within the mammalian cell, primarily controlling proliferation and survival, therefore the inhibition of one of these pathways prompting the activation of the other may serve as an evolutionary safeguard preventing cell death. As suggested by this current study, as well as work by Irie et al (2005), the compensatory mechanism that exists between the Akt and ERK pathways is not restricted to regulation of apoptosis and survival but also impacts on the regulation of migration.

It has previously been shown that S1PR1 activation can induce significant migration in astrocytes (Mullershausen et al. 2007) but the intracellular signalling pathways involved had not been examined till now. Based on the data in this chapter, it is likely that the phosphorylation of Akt is integral to the regulation of S1PR1-induced migration in astrocytes. S1PR1-induced migration is attenuated by the inhibition of Akt by MK2206, while both U0126 and MNP301 inhibit pERK but promote migration. The ability of U0126 to induce Akt phosphorylation and both U0126 and MNP301 to potentiate the S1PR1-induced phosphorylation of Akt, indicates that the activity of ERK, may exert tonic inhibition on the Akt pathway. U0126 and MNP301 downregulate pERK, thus reducing the inhibition of Akt, allowing for increased phosphorylation of Akt. A functional consequence of this decreased inhibition is seen in enhanced astrocyte migration. Constitutive S1PR1 activity, similar to that described in HEK cells (Waters et al. 2006), may explain why S1PR1 agonism is not necessary for MNP301 to promote migration. It may be the case that constitutive S1PR1 activity induces low level ERK phosphorylation that is inhibited by MNP301, thus increasing pAKT and increasing astrocyte migration. However, this hypothesis requires investigation and corroboration.

Regulation of astrocyte migration could have implications in disease states where astrogliosis is often mentioned as a contributing factor to impaired remyelination in the CNS. Conversely, increased motility of astrocytes would allow for chemotaxis to areas of damage, thus aiding recovery through the release of trophic factors and by providing structural repair.

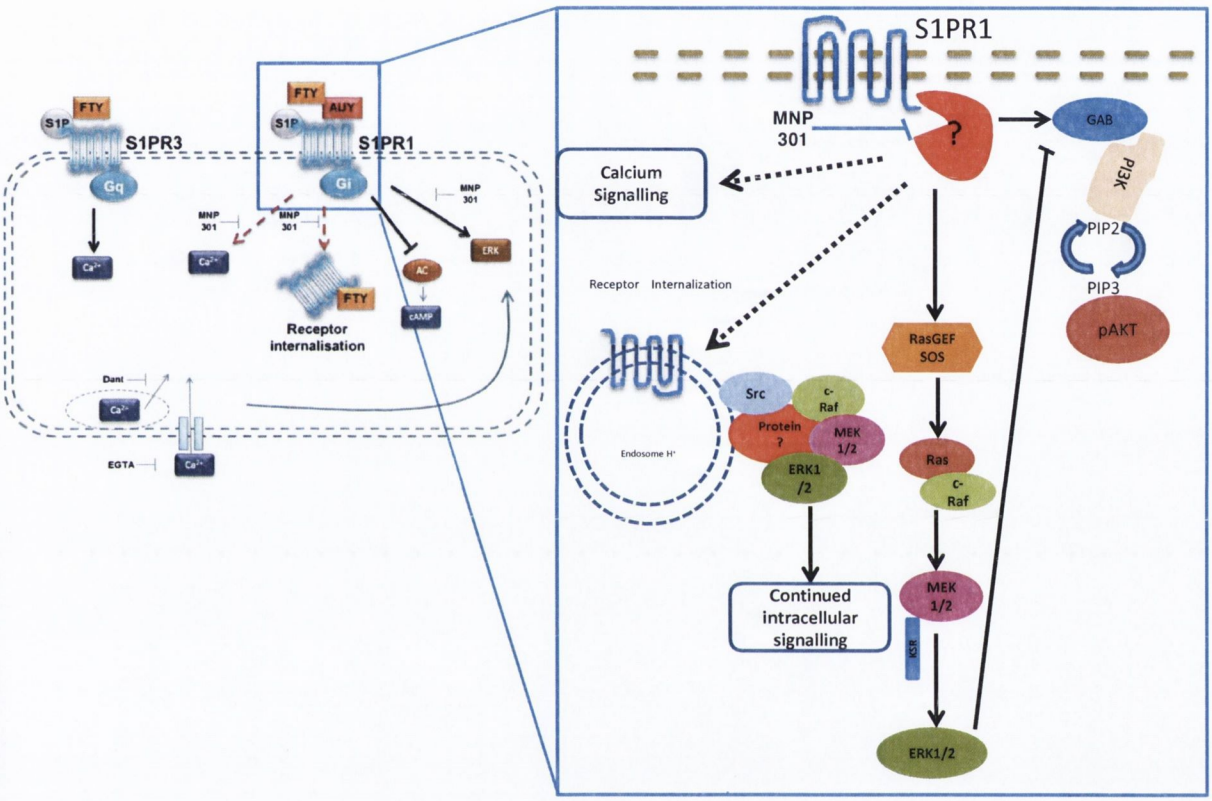

Figure 4.13 MNP301 inhibits ERK phosphorylation and potentiates activation of Akt

MNP301 inhibits an unidentified protein-protein interaction involving S1PR1. In this way it inhibits receptor internalisation, calcium signalling and ERK phosphorylation. Inhibition of ERK removes negative feedback on the Akt pathway thus enhancing pAkt levels.

Chapter 5. The effects of S1PR modulation in an immune cell-induced demyelination model

Chapter Aims:

- To validate a MOG-reactive splenocyte/organotypic cerebellar culture demyelination assay.

- To examine the effects of pFTY720 on the ability of MOG-reactive splenocytes to induce demyelination in this co-culture assay.

- To explore the effects of pFTY720 on the ability of 2D2 transgenic immune cells to induce demyelination in our assay

- To assess any changes in T cell phenotype due to application of pFTY720 to an i*n vitro* culture.

- To measure the relative release of cytokines from pFTY720 treated and non-treated MOG-reactive 2D2 cells.

Abstract

The family of sphingosine-1-phosphate receptors (S1PRs) is G-protein-coupled, comprised of subtypes S1PR1-S1PR5 and activated by the endogenous ligand S1P. The phosphorylated version of Fingolimod (pFTY720), an oral therapy for multiple sclerosis (MS), induces S1PR1 internalisation in T cells, subsequent insensitivity to S1P gradients and sequestering of these cells within lymphoid organs, thus limiting immune response. S1PRs are also expressed in neuronal and glial cells where pFTY720 is suggested to directly protect against lysolecithin-induced deficits in myelination state in organotypic cerebellar slices. Of note, previous studies in this labhave found that such slice cultures contain immune cells, which could also be regulated by pFTY720 to maintain levels of myelin. Here, a mouse organotypic cerebellar slice and lymphocyte co-culture model was used to investigate the effects of pFTY720 on lymphocyte-induced demyelination. Spleen cells isolated from MOG-immunised mice (MOG-lymphocytes) or from 2D2 transgenic mice (2D2-lymphocytes) both induced demyelination when co-cultured with mouse organotypic cerebellar slices, to a similar extent as lysolecithin. As expected, *in vivo* treatment of MOG-immunised mice with FTY720 inhibited demyelination induced by MOG-lymphocytes. Importantly, *in vitro* treatment of MOG- and 2D2-lymphocytes with pFTY720 also attenuated demyelination caused by these cells. In addition, while *in vitro* treatment of 2D2-lymphocytes with pFTY720 did not alter cell phenotype, pFTY720 inhibited the release of the pro-inflammatory cytokines such as IFN-γ and IL-6 from these cells. This work suggests that treatment of lymphocytes by pFTY720 attenuates demyelination and reduces pro-inflammatory cytokine release, which likely contributes to enhanced myelination state induced by pFTY720 in organotypic cerebellar slices.

1 Introduction

MS is believed to be an auto-immune disease that is driven by both innate and adaptive arms of the immune system. To develop effective therapies against MS, it is crucial to understand the underlying immune pathology. T cells are the key players in adaptive immunity and, once mature, they can be broadly split in to two groups; CD8 and CD4 co-receptor expressing cells.

1.1 $CD8^+$ T cells

Naive $CD8^+$ cells differentiate into cytotoxic T cells and are important for defence against intracellular pathogens such as viruses. These cells can be activated directly by mature dendritic cells that trigger the synthesis of interleukin-2 (IL-2) that drives their own proliferation and differentiation (Murphy et al. 2008). However, a co-stimulatory $CD4^+$ T cell is generally required to increase the activation of the virus-infected antigen-presenting cell to the level that is necessary to activate the $CD8^+$ cell (**Figure 5.1**). $CD4^+$ cells may also contribute to $CD8^+$ differentiation and proliferation through their production of IL-2. Once activated the cytotoxic $CD8^+$ cells can kill target cells that display peptide fragments of cytosolic pathogens bound to major histocompatibility complex class I (MHC I)molecules (**Figure 5.1**). They also release IFN-γ which reduces viral replication and may eliminate the pathogen from infected cells without killing them (Murphy et al. 2008).

1.2 $CD4^+$ T cells

Naive $CD4^+$ cells have the possibility of differentiating into a much wider variety of effector cells including: Th1, Th2, Th17, and T regulatory cells (T reg) (**Figure 5.2**). The presence of IFN-γ and IL-12 during the early stages of activation pushes the $CD4^+$ cell along the Th1 pathway (Murphy et al. 2008). These Th1 cells recognise bacterial antigens on the surface of macrophages and interact with them through the major histocompatibility complex class II (MHC II) molecules. They then produce cytokines, e.g. IFN-γ and TNF-α, that activate macrophages and allow them to destroy intracellular microorganisms more effectively. Th1 cells can also activate B cells to produce antibodies against extracellular pathogens (**Figure 5.2**), in particular IgG antibodies (Murphy et al. 2008).

The development of Th2 cells is reliant on the presence of the cytokine IL-4. These cells also drive the antibody production of B cells but these antibodies are of the IgE class responsible for fighting parasitic infection and for allergies. Th2 cells secrete IL-

10, an anti-inflammatory cytokine that, among other functions, can inhibit the activation of macrophages (**Figure 5.2**) (Murphy et al. 2008).

Th17 cells are a more recently identified phenotype. They recruit neutrophils to the site of infection through the secretion of IL-17, IL-6, TNF-α and the chemokine CXCL1. (**Figure 5.2**) (Murphy et al. 2008). Their role in the disease process of MS has become clearer in recent times and the importance placed upon this activity has increased greatly. There is some evidence to suggest that Th17 cells may form an immune-synapse by binding to and interacting with neurons in the CNS of EAE mice. In this manner the Th17 cells bring about death of these neurons *in vitro* (Siffrin et al. 2010). The relevance of this observation to MS is as yet, unsubstantiated, but it is a novel mechanism through which invading immune cells may impact on the CNS.

Treg cells function as suppressors of auto-immunity by regulating the activity of effector and cytotoxic T cell populations (Fontenot et al. 2003). They are committed to their role early in their development, before they leave the thymus. They are $CD4^+$ and also express the high affinity α chain of the IL-2 receptor (CD25) as well as the transcription factor Foxp3 (Pandiyan et al. 2007). T regs suppress immune responses but the mechanism by which they do so is unclear. It may be that physical contact between the Tregs and the effector cell is necessary for the suppression of activity or the Treg may secrete IL-10 and TGF-β and bring about suppression in this way. However, IL-2 is a pro-survival for these Tregs but as expression of Foxp3 down-regulates IL-2 production they do not produce IL-2 (Pandiyan et al. 2007) it therefore appears that the regulatory effects of these cells is due to their uptake of local IL-2 and deprivation of said cytokine for other cells (Pandiyan et al. 2007). A dysfunction in these T regs can lead to a variety of autoimmune diseases and tumours (Sakaguchi 2005).

1.3 S1PRs in Multiple Sclerosis

S1PRs, in particular the S1PR1 subtype, have been described over the last decade as important modulators of immune cell migration (Matloubian et al. 2004; Pappu et al. 2007; Zhi et al. 2011). Recently, the orally available S1PR agonist, FTY720, has shown efficacy in the treatment of relapsing remitting multiple sclerosis, supporting the use of S1PRs as bona fide drug targets (Kappos et al. 2010). It has been suggested that pFTY720 internalises S1PR1s to cause sequestration of T cells within the lymph nodes (Mandala et al. 2002; Matloubian et al. 2004) likely preventing S1P-dependent T cell transmigration into the peripheral circulation and consequently into the CNS.

Notably, however, reports investigating the effects pFTY720-mediated internalisation of S1PR1s on the activation state of the lymphocytes or related cytokine release require further elucidation. For example, some studies show that a subpopulation of T regs may be functionally augmented by pFTY720 (Fontenot et al. 2003; Fantini et al. 2007; Sehrawat & Rouse 2008; Sun et al. 2011), which has been suggested as potentially beneficial in autoimmune or inflammatory illnesses. In contrast, others put forward the idea that pFTY720 may prevent the proliferation of T regs and functionally impair them (Wolf et al. 2009). Thus, further studies examining the effects of pFTY720 on these T cell subpopulations may prove useful.

There is now a growing body of evidence to support that S1PRs also play a number of roles in regulating the physiology of neuronal and glial cells in the CNS (Dev et al. 2008; Pritchard & Dev 2013). With regard to oligodendrocyte function and myelination state, many studies have reported the positive effects of pFTY720 on both these processes, where S1PRs are suggested to play roles in remyelination as well demyelination (Pritchard & Dev 2013). The first of these studies elegantly described how pFTY720 increased remyelination 14 days after lysolecithin (LPC)-induced demyelination, which was suggested to be driven via S1P3R/S1P5R, with S1PR1 limiting remyelination (Miron et al. 2010). Sheridan and Dev (2012) then showed using rat organotypic cerebellar slice cultures, that pFTY720 and SEW2871 (a S1PR1-specific agonist) also inhibited LPC-induced demyelination as assessed by MBP immunofluorescence. In that study, it was reported both pFTY720 and SEW2871 inhibited the release of several chemokines in conditions of LPC-induced demyelination, including LIX (CXCL5), MIP-1alpha, and MIP-3alpha (Sheridan & Dev 2012). It was also observed that the organotypic slice cultures stained positive for a number of immune cells (Sheridan & Dev 2012), as previously reported by others (Ling et al. 2008; Prodinger et al. 2011). This finding raised the question whether pFTY720 attenuated demyelination by reducing pro-inflammatory response of these 'brain-slice resident' immune cells and/or by directly altering neuronal and/or glial cell function.

One of the challenges of investigating the role of S1PRs in oligodendrocytes on myelination has been the limitation to assess oligodendrocyte function in the context of inflammatory models. The specific knockout of S1PR1 from oligodendrocytes appears to increase sensitivity to cuprizone-induced demyelination (Kim et al. 2011); although no deficits in myelination state has been reported for S1PR5-null mice (Jaillard et al. 2005). In cuprizone and LPC models of *in vivo* demyelination, FTY720 has not been shown to rescue myelination state, although FTY720 has been shown to attenuate

cuprizone-induced damage to oligodendrocytes in the corpus callosum (Kim et al. 2011; Hu et al. 2011). Similar to the reported *in vitro* studies (Sheridan & Dev 2012; Jackson et al. 2011); these protective effects of FTY720 are associated with a reduction in pro-inflammatory cytokines and chemokines (Kim et al. 2011). From these studies, it appears that pFTY720 promotes myelin repair by likely modulating both S1PRs expressed in immune and glia cells, although the relative contribution of each cell type remains to be fully established.

1.4 Aims and Hypothesis

There is much debate as to whether the effects exerted by FTY720 within the CNS are beneficial to MS therapy or whether the majority of benefits of the compound are due to lymphocyte sequestration in the lymph nodes. Here, this chapter aims to explore a third option, the effect that pFTY720 may have on peripheral immune cells that are reactive to myelin proteins but have already migrated into the CNS prior to initiation of treatment. In order to examine this, an assay will be utilised, whereby MOG-reactive splenocytes are co-cultured with organotypic cerebellar slices in order to induce demyelination. Therefore the experimental model will not be dependent on chemical-induced demyelination. In this manner, the direct effects of pFTY720 on immune cell ability to induce demyelination will be investigated.

We hypothesise that splenocytes, reactivated in the presence of pFTY720 *in vitro,* will have reduced inflammatory product expression, possibly due to a change in cell phenotype, and thus an impaired ability to induce demyelination in a cerebellar slice co-culture.

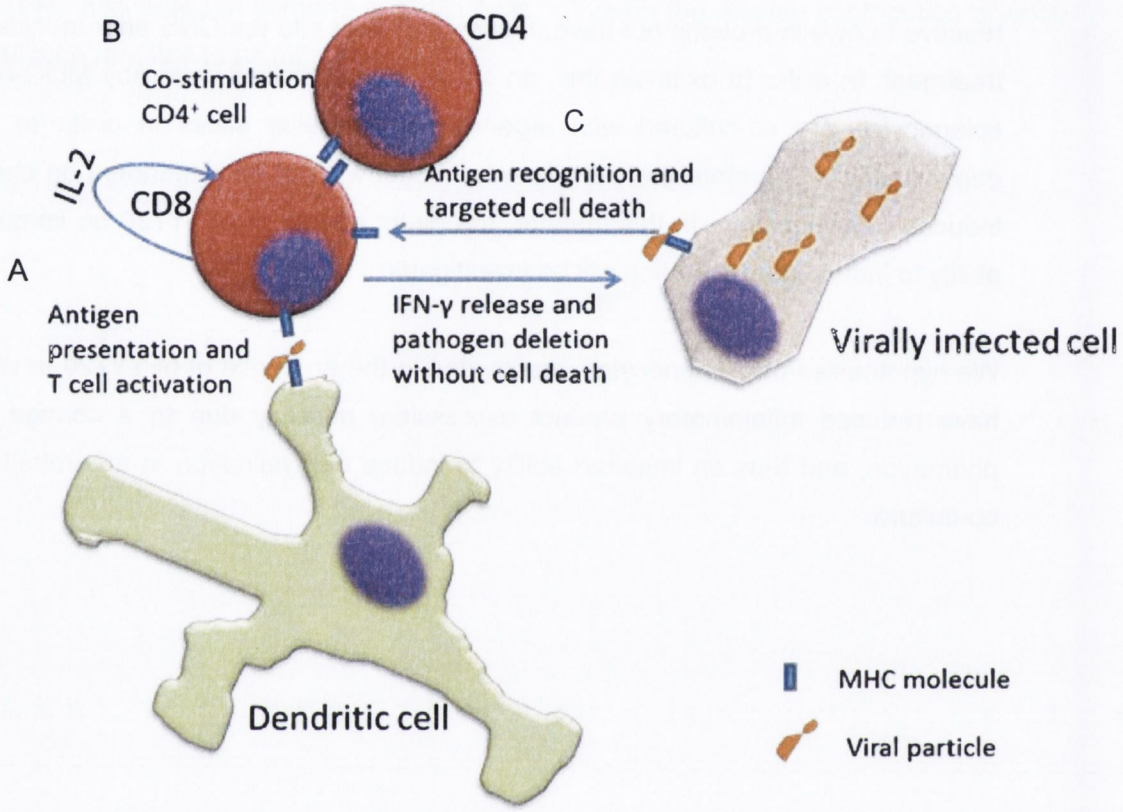

Figure 5.1 T cell activation.

(A) Dendritic cells present viral particles to naïve $CD8^+$ cells in the presence of co-stimulation **(B)** from $CD4^+$ cells. The newly activated $CD8^+$ cells releases IL-2 causing autocrine signalling through IL-2 receptors. The T cell can then bring about targeted cell death **(C)** of virally infected cells or delete the pathogen through IFN-γ release.

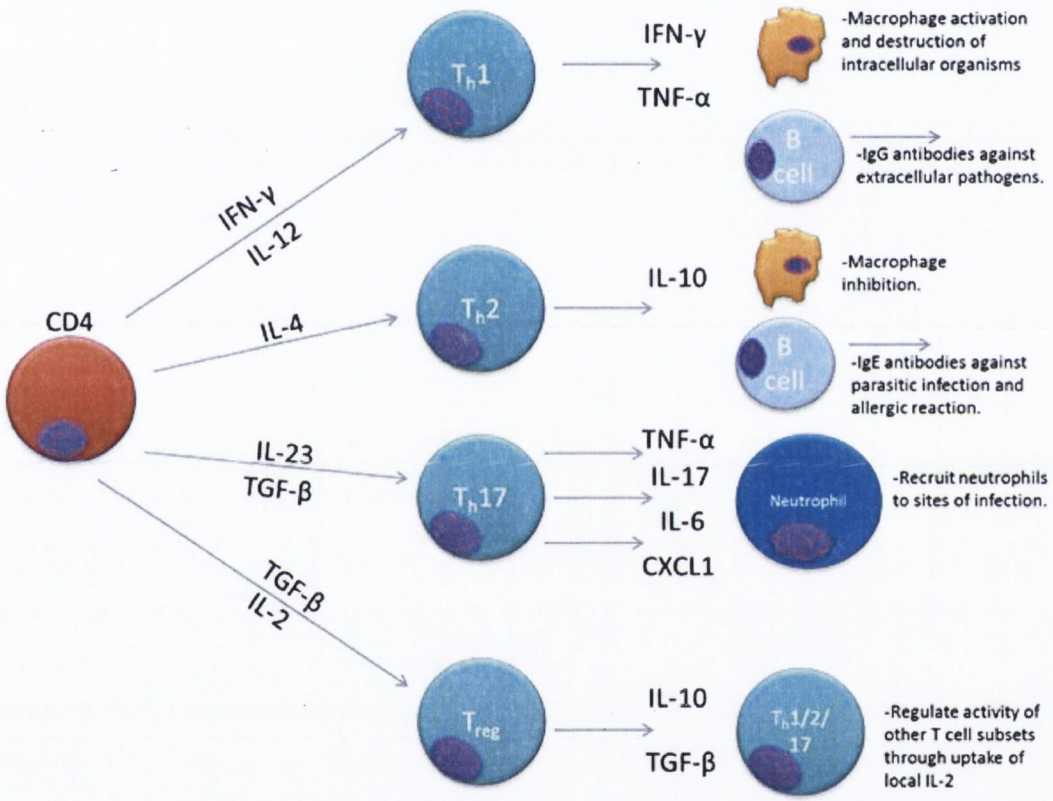

Figure 5.2 CD4$^+$ T cell lineage and post-activation function.

Naïve CD4$^+$ T cells, activated in the presence of different cytokines, differentiate into varied T cell phenotypes and allow for the diverse activities associated with adaptive immunity.

2 Results

2.1 Myelination increases over time in cerebellar slice cultures

Prior to examining the effects of S1PR modulation on lymphocyte-induced demyelination, the development and organization of neuronal fibres and myelin under control conditions, as determined by neurofilament and MBP expression, respectively, was examined in mouse organotypic cerebellar slice cultures. An approximate 2-fold increase in myelination between day 4 *in vitro* (DIV4) and DIV9 ($p<0.05$, one-way ANOVA and Newman-Keuls post-hoc test) was found (**Figure 5.3**). Myelination continued beyond DIV9, where an approximate 6-fold increase in MBP expression was found at DIV12 compared to DIV4 ($p<0.001$, one-way ANOVA and Newman-Keuls post-hoc test). At this later time point, MBP and neurofilament staining was found predominantly colocalised along the Purkinje cell axons, indicative of axons displaying an intact myelin sheath (**Figure 5.3**). Based on this information, co-culture of slices and lymphocytes was initiated at DIV12.

2.2 Splenocytes isolated from MOG-immunised mice induce demyelination

Lysophosphatidylcholine (LPC) is a chemical compound that induces significant demyelination *in vitro.* As a positive control, slices were treated with LPC (350 µg/ml for 18h) (Miron et al. 2010; Sheridan & Dev 2012) inducing a significant demyelination (52.7% ± 12.6% SEM) compared to untreated control ($p<0.01$, one-way ANOVA and Newman-Keuls post-hoc test) and confirming previous findings (Sheridan and Dev 2012) (**Figure 5.4**). Next splenocyte-induced demyelination was examined in these organotypic cerebellar slice cultures. It has recently been shown that lymphocytes isolated from nodes of wild-type mice (naïve-lymphocytes) are not detrimental to organotypic hippocampal slice cultures, and indeed are neuroprotective against toxicity induced by kainate-and oxygen-glucose deprivation (Shrestha et al. 2014). Accordingly, naïve splenocytes activated by anti-CD3/CD28 did not induce any significant change in MBP staining when applied to organotypic cerebellar slice cultures (**Figure 5.4**). In contrast, cells isolated from spleen of MOG-immunised mice (MOG-splenocytes), re-stimulated *in vitro* with MOG_{35-55} (25 µg/ml for 48h) and co-cultured with cerebellar slices for 48h (**Figure 5.4**) induced a significant decrease in MBP staining (59.2% ± 8.0% SEM) compared to naïve-splenocytes ($p<0.05$, one way ANOVA and Newman-Keuls post-hoc test) (**Figure 5.4**). Overall, these results support this lab's previous data and data published by others and demonstrate the amount of

demyelination induced by MOG-lymphocytes is comparable to that seen in LPC treated slices.

2.3 *In vivo* and *in vitro* treatment with FTY720 attenuates demyelination induced by MOG splenocytes

Reports have shown that FTY720 can reduce the pro-inflammatory response of immune cells or induce anti-inflammatory cell phenotypes (Sehrawat & Rouse 2008). As a positive control and to further confirm the validity of this splenocyte-induced demyelination model, the ability of FTY720 in attenuating demyelination induced by MOG-splenocytes was examined. The MOG-immunised mice were co-treated with FTY720 *in vivo* (8 µg/mouse/day for 10 days) and isolated MOG-splenocytes were also treated with pFTY720 *in vitro* (1nM for 48h) during re-stimulation with MOG_{35-55} peptide (25µg/ml for 48h) before co-culture with organotypic cerebellar slices. Confocal images showed significant reduction in MBP expression in slices treated with MOG-lymphocytes (65.2% ± 7.9% SEM), compared to controls ($p<0.05$, one way ANOVA and Newman-Keuls post-hoc test). Not surprisingly, MOG lymphocytes treated with FTY720 (94.0% ± 11.1% SEM) displayed no difference in MBP expression compared to control (**Figure 5.5**). In addition, Western blotting for MOG protein showed a decrease in MOG expression in cerebellar slices treated with MOG-reactive cells compared to controls and as expected FTY720 attenuated the effects of MOG-reactive cells (**Figure 5.5**). This data indicates that *in vivo* treatment of MOG-immunised mice with FTY720 followed by *in vitro* treatment of MOG-lymphocytes with pFTY720 reduces the ability of these MOG-lymphocytes to induce demyelination.

2.4 *In vitro* treatment of MOG-splenocytes with pFTY720 is sufficient to attenuate their capacity to induce demyelination

The experiment detailed above shows that cells isolated from MOG-immunised mice treated with FTY720 *in vivo* and then pFTY720 *in vitro* no longer induce demyelination. To elucidate whether pFTY720 had direct effects on the ability of these MOG-reactive cells to demyelinate *in vitro*, cells were isolated from MOG-immunised mouse spleens and activated with MOG_{35-55} peptide (25 µg/ml for 48h) in the presence or absence of pFTY720 (1nM) before co-culture with organotypic cerebellar slices. Confocal imaging showed that the addition of MOG-splenocytes to cerebellar slices induced a significant level of demyelination compared to control (55.85% ± 2.4% SEM) ($p<0.01$, one-way ANOVA and Newman-Keuls post-hoc test) (**Figure 5.6**). However, MOG-splenocytes

activated in the presence of pFTY720 did not show a significant reduction in myelination (84.7% ± 3.25% SEM) based on MBP expression. Therefore, this experiment shows that *in vitro* treatment of MOG-reactive cells with pFTY720 can reduce their ability to induce demyelination.

2.5 *In vitro* treatment of splenocytes isolated from 2D2 transgenic mice with pFTY720 attenuates demyelination

In the experiments above splenocyte cultures were used, which were treated and then cocultured with cerebellar slices. To confirm the specific reactivity of these cells splenocytes were isolated from the spleens of 2D2 transgenic mice (2D2-lymphocytes), which transgenically express a T cell receptor for MOG (TCR-MOG) and are thus engineered to be stimulated by MOG. Rather than artificially isolating lymphocytes from other cell types, likely limiting them from cellular crosstalk, it was decided to specifically stimulate this cell-type in splenocyte cultures. The effects of pFTY720 on demyelination induced by these MOG-reactive 2D2-lymphocytes in organotypic cerebellar slices were then examined. The treatment of organotypic cerebellar slices with these 2D2-splenocytes induced demyelination, similar to MOG-splenocytes and LPC treatments (**Figure 5.7**). MBP staining showed a significant reduction in slices treated with 2D2-lymphocytes (51.6% ± 6.3% SEM) compared to control ($p<0.05$, one way ANOVA and Newman-Keuls post-hoc test). Importantly, the *in vitro* treatment of 2D2-splenocytes with pFTY720 (1 µM for 48h) during re-stimulation with MOG_{35-55} (25 µg/ml for 48h) before co-culture with organotypic cerebellar slices, attenuated the ability of these cells to induce demyelination (82.5% ± 5.2% SEM) Western blotting for MOG protein confirmed these findings, and demonstrated a decrease in MOG expression in cerebellar slices treated with 2D2-lymphocytes compared to controls, whereas pFTY720 attenuated the effects of 2D2-lymphocytes to induce demyelination (**Figure 5.7**). This data suggests that pFTY720 treatment *in vitro* diminishes the ability of lymphocytes to induce demyelination.

2.6 pFTY720 treatment of 2D2-splenocytes does not alter the T cell phenotype

Studies have proposed that pFTY720 exerts anti-inflammatory effects on T cells, possibly by promoting T reg response, while reducing Th17 cell-mediated inflammation. For example, pFTY720 increases in the proportion of T regs *in vivo* and *in vitro* (Daniel et al. 2007; Sehrawat & Rouse 2008; Kim et al. 2011), while attenuating S1P-induced expansion of Th17 cells and consequent release of IL-17 (Liao et al.

2007). Some of these studies have however suggested that pFTY720 reduces numbers of T regs (Wolf et al. 2009). In order to ascertain whether *in vitro* treatment of 2D2-splenocytes with pFTY720 altered their phenotype, cells were stimulated with MOG_{35-55} peptide in the presence or absence of this drug. No significant change in $CD4^+$ cell population was observed with pFTY720 treatment (**Figure 5.8**). The addition of IL-2, in conjunction with pFTY720, also had no effect on the $CD4^+$ percentage (data not shown). The effects pFTY720 on activated $CD4^+$ T cell populations were also examined. No significant change was observed in the proportion of $CD4^+CD25^+$ cells in response to pFTY720 treatment in MOG_{35-55} peptide stimulated 2D2 cells either without (**Figure 5.8**) or with IL-2 (data not shown). To further examine the effect of pFTY720 on T regs, cells positive for $CD25^+Foxp3^+$ were examined. Treatment of 2D2-splenocytes stimulated with MOG_{35-55} peptide with or without pFTY720 did not alter T reg proportion either in the absence (**Figure 5.8**) or presence of IL2 (data not shown). In contrast, treatment of 2D2-splenocytes with TGF-β increased the percentage of T regs by up to 20 fold, as expected (**Figure 5.8**). Based on these *in vitro* results, it appears that pFTY720 does not alter T reg populations within a mixed culture and is unlikely to explain the effects of this drug in limiting demyelination caused by 2D2-transgenic cells in organotypic cerebellar slices.

2.7 pFTY720 significantly reduces pro-inflammatory cytokine release by 2D2-splenocytes *in vitro*

IFNγ, TNFα and IL6 are reported as pro-inflammatory cytokines that play a variety of roles in EAE and MS disease state (Akassoglou et al. 1998; Murphy et al. 2010; Schneider et al. 2013). In addition, levels of IL10 secreting mononuclear cells have been shown to be reduced in MS sufferers, (Ozenci et al. 1999), likely indicating impaired anti-inflammatory function. An attenuation of the levels of these pro-inflammatory cytokines, without an impairment of anti-inflammatory cytokine release, would likely have numerous beneficial effects in myelination state. Previous studies have shown that S1P attenuates the production of IFNγ by splenic $CD4^+$ T cells, and this effect is likely mediated via S1PR1, although the direct effects of pFTY720 were not tested at that time (Dorsam et al. 2003; Liao et al. 2007). Moreover S1PRs also regulate the release of TNF-α and IL6 from cells types such as dendritic cells, macrophages and microglia (Oz-Arslan et al. 2006; Noda et al. 2013; Potì et al. 2013a). It was found that pFTY720 did not significantly alter IL10 levels in 2D2-splenocytes stimulated with MOG_{35-55} peptide (**Figure 5.9**). In contrast, pFTY720 reduced the levels of IFNγ in a concentration dependent manner, where 2D2-

splenocytes stimulated with MOG_{35-55} peptide in the presence of pFTY720 (1μM) displayed a significant reduction in levels of IFNγ (122.2% ± 20% SEM) compared to MOG-stimulated control (304% ± 50.8% SEM, $p<0.01$, one-way ANOVA and Newman-Keuls post-hoc test) (**Figure 5.9**). pFTY720 (1μM) also significantly reduced the levels of IL6 (198% ± 18.8% SEM), compared to MOG-stimulated control (280.9% ± 18.5% SEM, $p<0.05$, one way ANOVA and Newman-Keuls post-hoc test) (**Figure 5.9**). The effect of pFTY720 on levels of TNFα in MOG_{35-55} peptide activated 2D2-splenocytes was moderate, with a trend reduction in levels of TNFα induced by pFTY720, which was not significant (**Figure 5.9**). Taken together, these data suggest that pFTY720 may attenuate 2D2-splenocyte mediated demyelination by limiting levels of pro-inflammatory molecules.

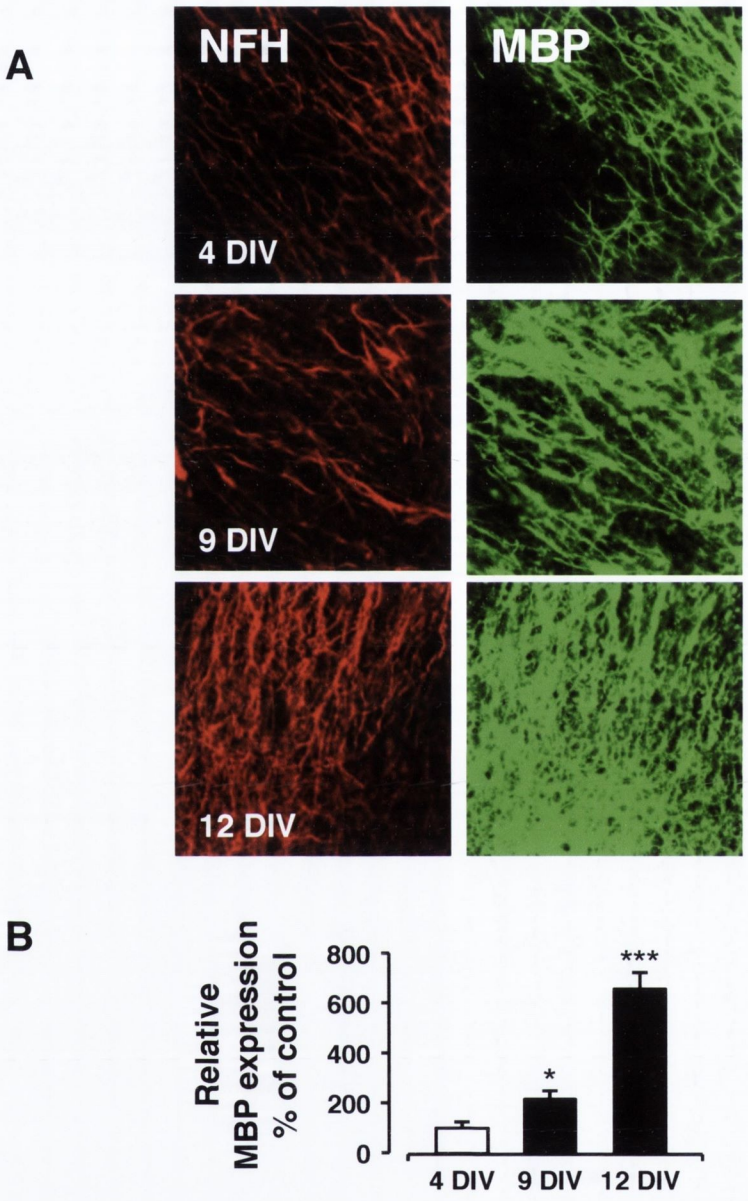

Figure 5.3 Myelination increases over time in cerebellar slice cultures

(A) Representative confocal images displaying MBP (MBP, green) and neurofilament (NFH, red) immunoreactivity at 4, 9 and 12 DIV. Confocal images captured at x40 magnification. **(B)** Bar graph illustrates MBP immunoreactivity in cerebellar slices relative to 4 DIV (100% as control) expressed as averages +/- SEM (12 slices per treatment group).

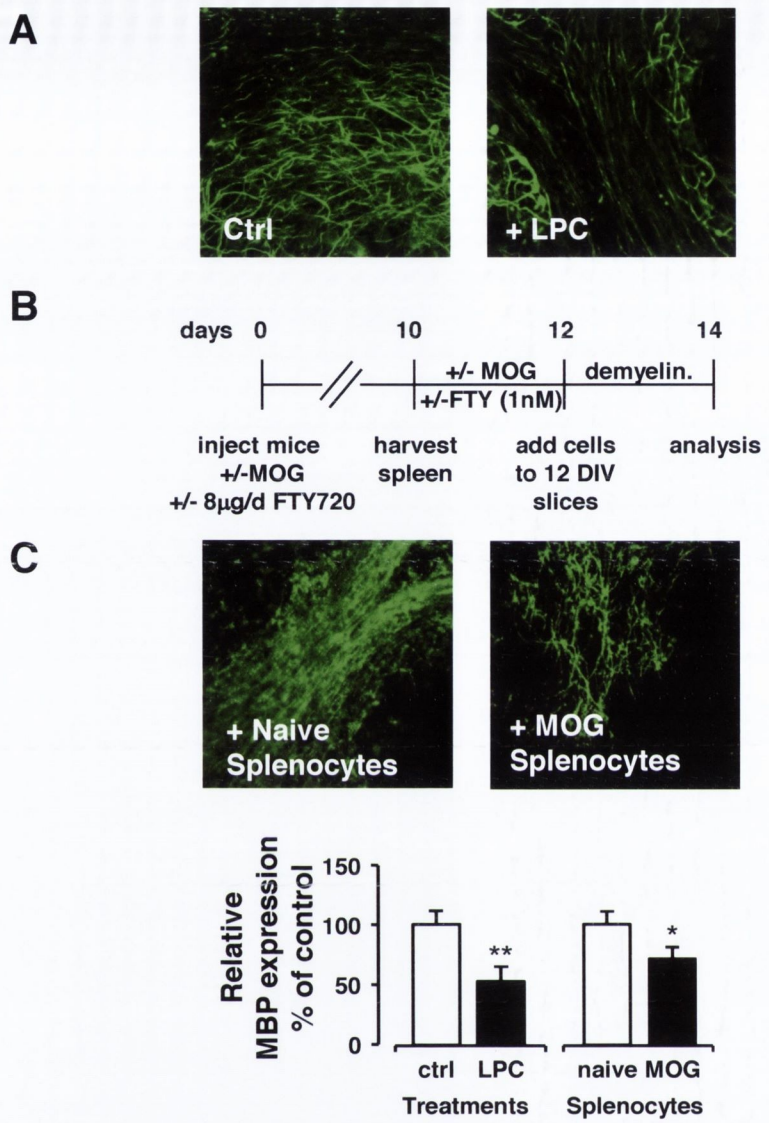

Figure 5.4 MOG-splenocytes induce demyelination comparable to LPC

(A)LPC-induced demyelination. MBP expression is reduced in cerebellar slices treated with LPC (350 mg/ml) as previously reported (Sheridan and Dev 2012). **(B)** Experimental setup. MOG-immunisation procedure and slice/splenocyte co-culture timeline is shown. **(C)** MOG reactive splenocytes induce demyelination. MBP expression is reduced in cerebellar slices treated with MOG reactive splenocytes (i.e. splenocytes isolated from MOG-immunised mice and re-stimulated *in vitro* with 25 µg/ml MOG), but not naive splenocytes isolated from control mice. Bar graph illustrates MBP immunoreactivity in cerebellar slices relative to no treatment (100% as control) expressed as averages +/-SEM (18-24 slices per treatment group). Significant difference *$p<0.05$, **$p<0.01$; One-way ANOVA and Newman-Keuls post-hoc test.

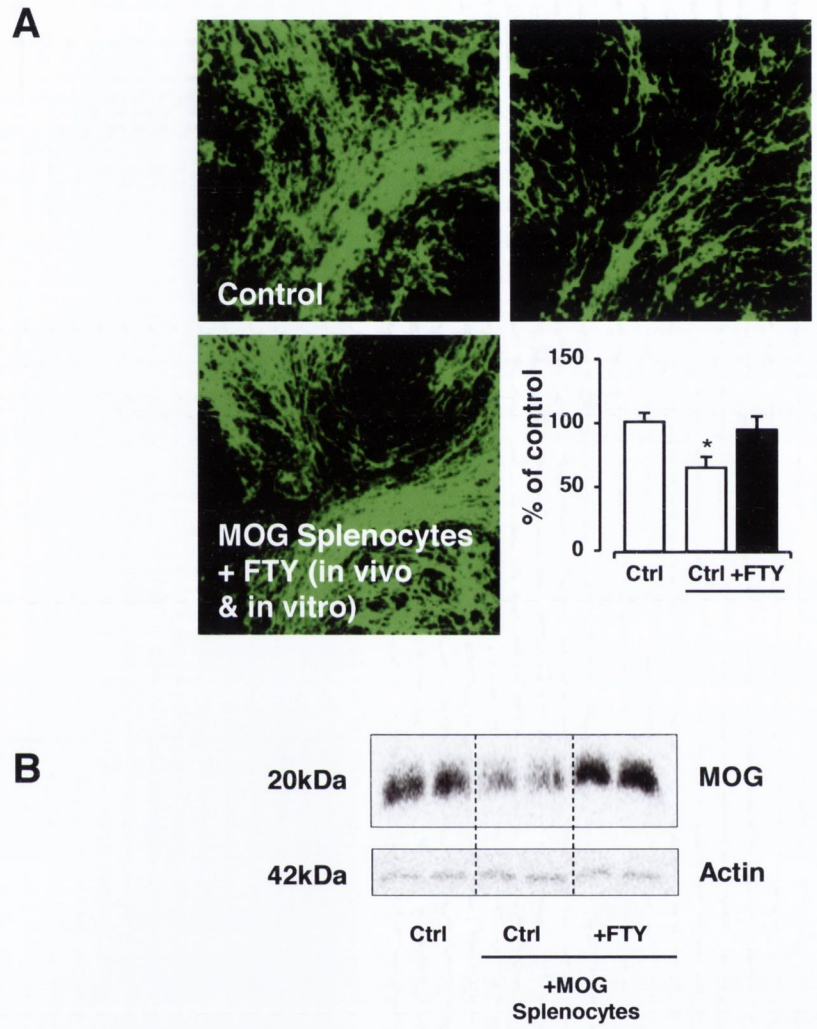

Figure 5.5 Treatment with pFTY720 *in vivo* and *in vitro* reduces MOG reactive splenocyte-induced demyelination

(A) MOG reactive reduced MBP staining in cerebellar slices. MOG reactive splenocytes isolated from MOG-immunised animals treated with pFTY720 (8 µg/day, for 10 days) *in vitro* and treated again with pFTY720 (1 nM) during re-stimulation *in vitro* with 25 µg/ml MOG prevented demyelination caused by these isolated cells. Bar graph illustrates MBP immunoreactivity in cerebellar slices relative to no treatment (100% as control) expressed as averages +/- SEM (20 slices per treatment group). Significant difference *$p<0.05$ from control; one-way ANOVA and Newman-Keuls post hoc test **(B)** Western blot shows decreased levels of MOG protein in MOG reactive cell treated slices, with *in vitro/in vitro* pFTY720 treatment reducing demyelination

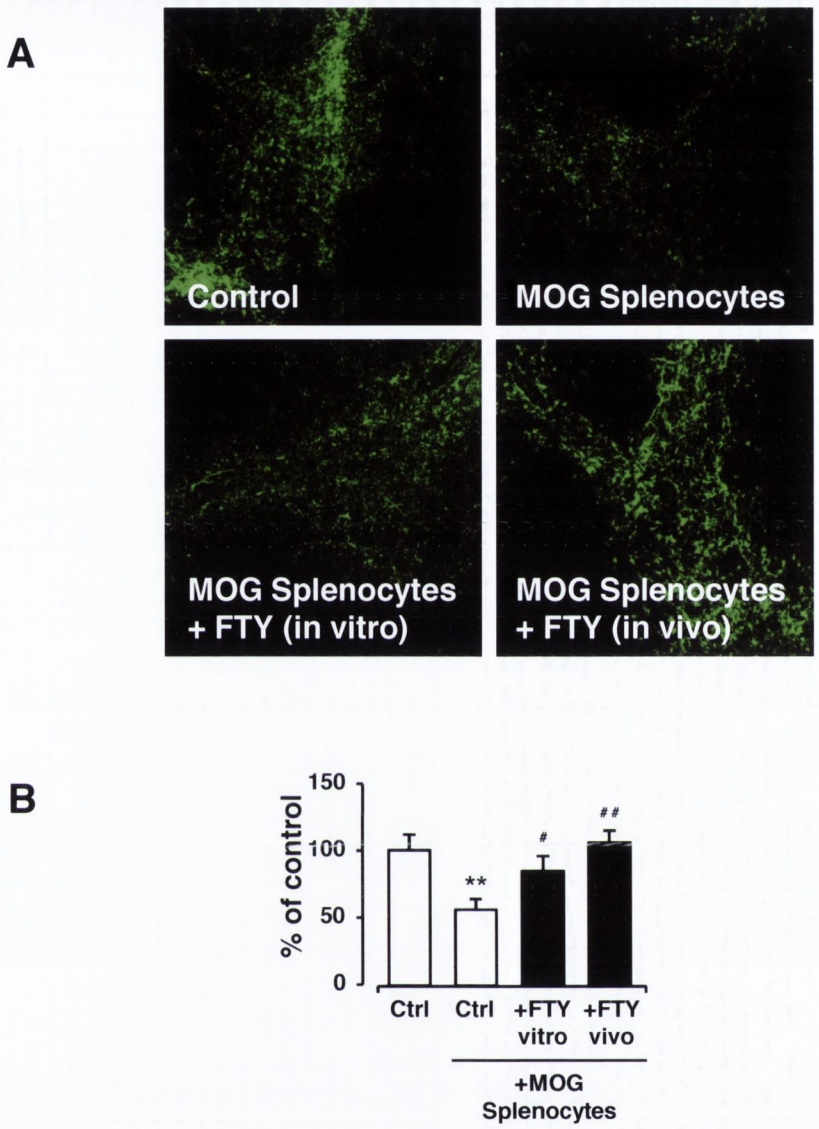

Figure 5.6 Treatment with pFTY720, either *in vitro* or *in vivo,* reduces MOG reactive splenocyte-induced demyelination.

(A) MOG reactive splenocytes isolated from MOG-immunised animals treated with pFTY720 (8 µg/day, for 10 days) *in vitro* or treated with pFTY720 (1 nM) during re-stimulation *in vitro* with 25 µg/ml MOG prevented demyelination caused by these isolated cells. **(B)** Bar graph illustrates MBP immunoreactivity in cerebellar slices relative to no treatment (100% as control) expressed as averages +/- SEM (20 slices per treatment group). Significant difference **p<0.01 from control; #p<0.05, ##p<0.01 from MOG-lymphocytes not treated with pFTY720; one-way ANOVA and Newman-Keuls post-hoc test.

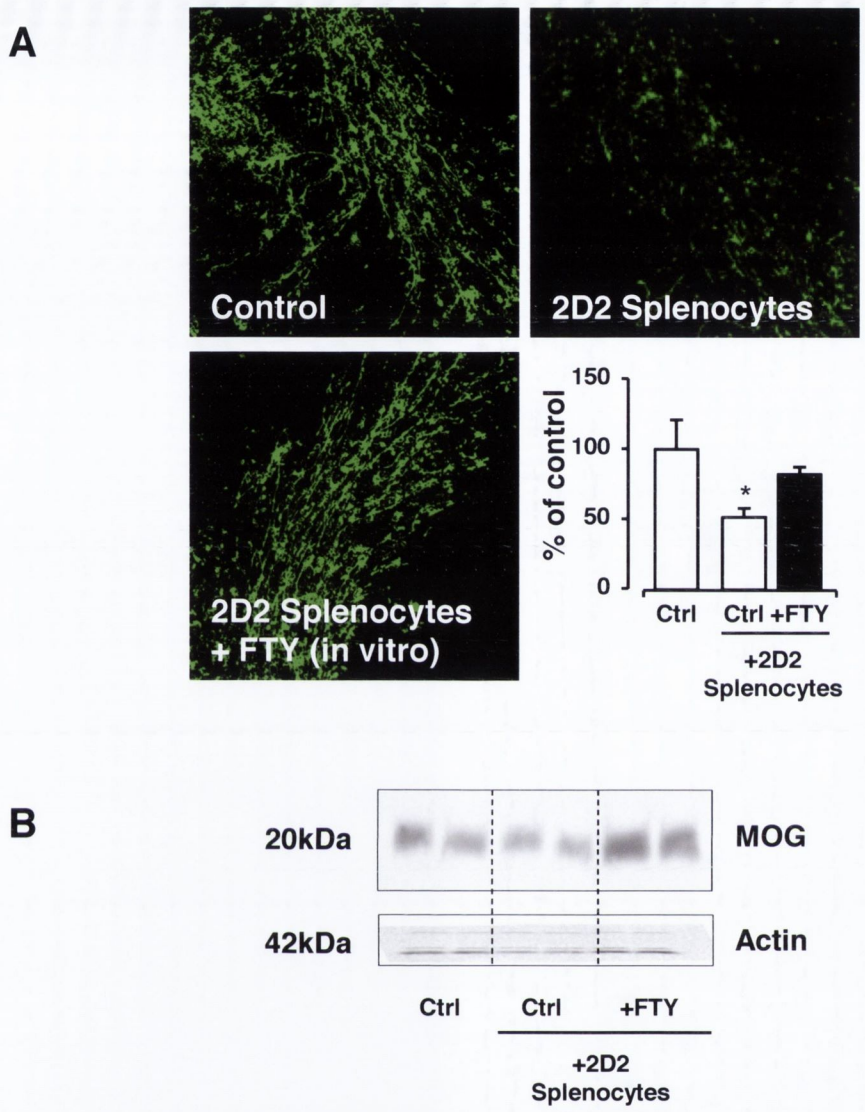

Figure 5.7 Treatment with pFTY720 *in vitro* reduces demyelination induced by 2D2-splenocytes.

(A) 2D2-lymphocytes (i.e. splenocytes isolated from 2D2 transgenic mice and stimulated *in vitro* with 25 µg/ml MOG) reduce MBP staining in cerebellar slices. 2D2- splenocytes isolated from 2D2 transgenic mice treated with pFTY720 (1 µM, 48hrs) during stimulation *in vitro* with 25 µg/ml MOG prevented demyelination caused by these isolated cells. Bar graph illustrates MBP immunoreactivity in cerebellar slices relative to no treatment (100% as control) expressed as averages +/- SEM (18-24 slices per treatment group). Significant difference *$p<0.05$ from control; One-way ANOVA and Newman-Keuls post-hoc test. **(B)** Western blot shows decreased MOG protein in 2D2-lymphocyte treated slices, with *in vitro* pFTY720 treatment reducing demyelination

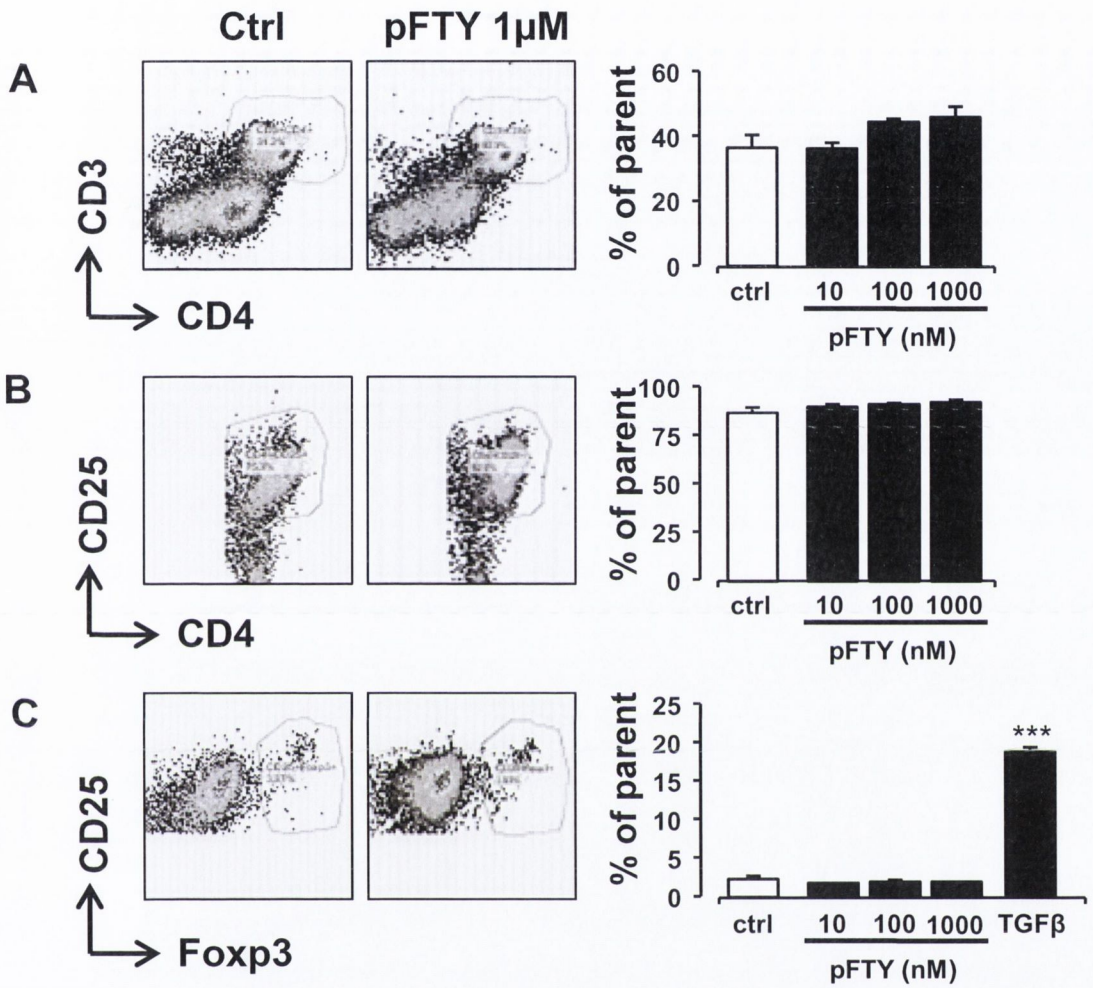

Figure 5.8 Treatment with pFTY720 *in vitro* does not alter phenotype of 2D2-splenocytes. Treatment with pFTY720 (10, 100, 1000 nM for 96hrs) of 2D2- splenocytes during stimulation with 25μg/ml MOG_{35-55} peptide did not alter the proportion of **(A)** $CD4^+$ cells, **(B)** $CD4^+/CD25^+$ cells, or **(C)** $CD4^+CD25^+Foxp3^+$ T reg cells. The addition of IL2 (20 ng/ml) in conjunction with pFTY720 (10, 100, 1000 nM) did not alter T reg cell frequency (data not shown). Bar graphs illustrate values expressed as averages +/- SEM (n = 4).Significant difference ***$p<0.001$ from control; One-way ANOVA and Newman-Keuls post-hoc test.

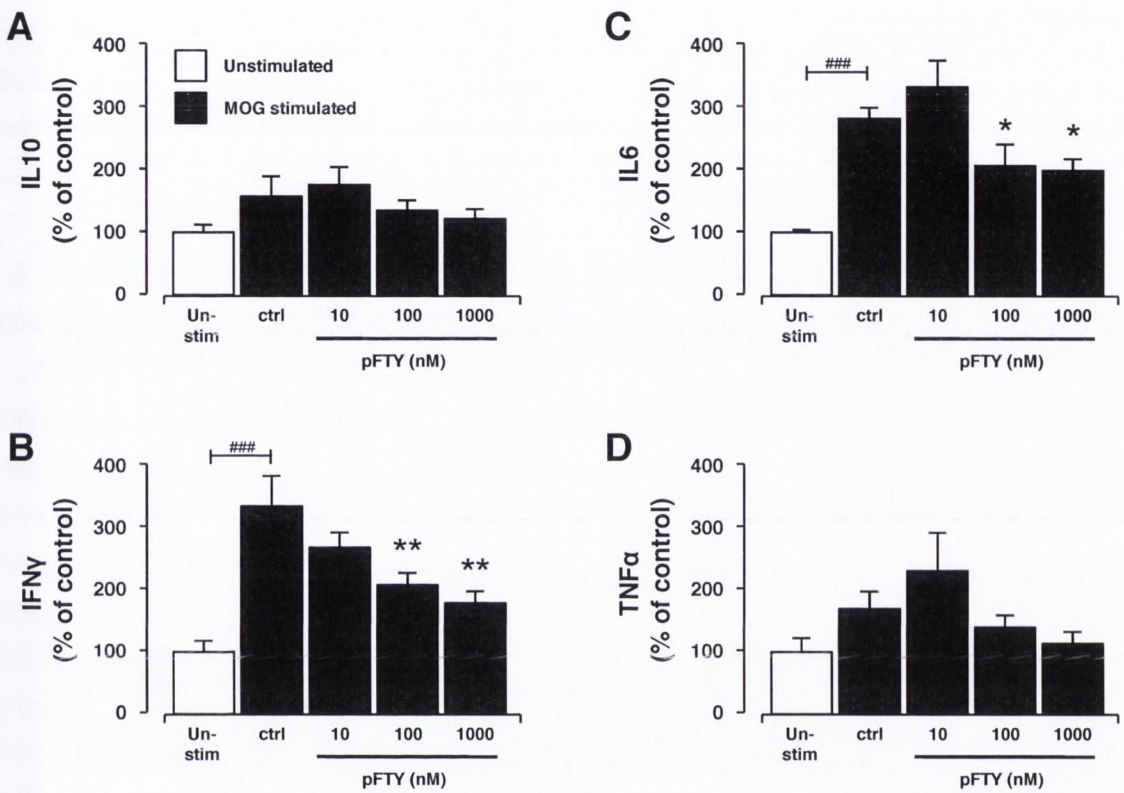

Figure 5.9 pFTY720 treatment *in vitro* reduces the release of pro-inflammatory cytokines from 2D2-splenocytes.

Treatment with pFTY720 (10, 100, 1000 nM for 96hrs) of 2D2-splenocytes during stimulation with 25µg/ml MOG_{35-55} peptide did not alter levels of **(A)** IL10 or, **(D)** TNFα but attenuated levels of **(C)** IFNγ, and **(B)** IL6. Bar graphs illustrate values expressed as averages +/- SEM (n = 4). Significant difference $^{###}p<0.001$ to unstimulated control; $^{*}p<0.05$, $^{**}p<0.01$ from stimulated control; One-way ANOVA and Newman-Keuls post-hoc test.

3 Discussion

3.1 Does S1PR modulation impair immune-cell-induced demyelination?

This lab and others have previously reported that pFTY720 promotes remyelination and attenuates demyelination in organotypic slice cultures (Miron et al. 2010; Sheridan & Dev 2012). During previous studies, it was noted that such organotypic slice cultures contain immune cells that may be modulated by pFTY720 during periods of remyelination or demyelination (Ling et al. 2008; Prodinger et al. 2011; Sheridan & Dev 2012). This finding raises the question whether pFTY720 attenuates demyelination by reducing pro-inflammatory response of these 'brain slice resident' immune cells and/or by directly altering neuronal and/or glial cell function. Thus, it was necessary to identify if pFTY720 limits demyelination in organotypic slice cultures by directly acting on brain cells or whether modulation of exogenously applied immune cells was sufficient to correct aberrant myelination state. Here, MOG splenocyte (isolated from MOG-immunised mice) induced demyelination in organotypic cerebellar slice cultures and, as expected, *in vitro* treatment of these MOG-immunised mice with FTY720 reduced the ability of these cells to induce demyelination. Importantly, the *in vitro* treatment of these MOG-splenocytes and 2D2-splenocytes (isolated from 2D2 transgenic mice) with pFTY720 also attenuated the ability of these cells to induce demyelination. While it was found that *in vitro* treatment of the 2D2-splenocytes with pFTY720 did not alter T cell phenotype, the data suggested that *in vitro* treatment of these cells with pFTY720 reduced levels of the pro-inflammatory cytokines, such as IFNγ and IL6.

3.2 Is this assay an effective model of immune-induced demyelination?

Taken together, three noteworthy points emerge from this current study: (i) firstly, this organotypic/ splenocyte model provides an assay to investigate mechanisms by which immune cells may induce demyelination, (ii) secondly, the treatment of MOG-reactive splenocytes with pFTY720 is sufficient to attenuate demyelination induced by these cells, and (iii) thirdly, a pFTY720-mediated decrease in the levels of pro-inflammatory cytokines, such as IFN-γ and IL-6 (and likely others) in immune cells likely plays a role by which this compound limits demyelination and promotes remyelination. In addition to these current findings, there are some important technical features of this study which are worthy of note. Firstly, in this lab and other's previous studies investigating the effect of pFTY720 on myelination state LPC, has been used to induce demyelination (Miron et al. 2010; Sheridan & Dev 2012). While previous studies have shown LPC to cause demyelination *in vitro* and *in vitro*, a concern using this agent is that the mechanism by which LPC induces demyelination is not fully clear. Therefore,

in the current study it was decided to further these previous findings using a cell-mediated demyelination model.

Secondly, in this current study, it should be noted that splenocyte cultures from MOG-immunised mice were used to induce demyelination, which were a mixed population of cells. To investigate the specific role of MOG-reactive immune cells, splenocyte cultures isolated from 2D2 transgenic animals were used. The cells isolated from these 2D2 mice contain lymphocytes engineered to express a T cell receptor activated by MOG (TCR-MOG) and can thus be stimulated by MOG to specifically generate MOG-reactive cells. This approach was used on the assumption that it would be important to generate MOG-reactive lymphocytes in a mixture of cells rather than isolating this individual cell subtype and removing it from environmental support.

Thirdly, it was noted that there was a possibility that pFTY720 added to the splenocyte cultures may be taken up by these cells and even after washing, this compound may persist and be carried over to the cerebellar culture, where it has been shown to have a direct effect on myelination (Sheridan & Dev 2012). This possibility, however, appears unlikely given previous studies showing that the phosphorylated from pFTY720 does not permeate lymphocytes, must be de-phosphorylated in order to enter the intra-cellular space, and then requires phosphorylation back to pFTY720 before leaving the cell and acting on receptors (Sensken et al. 2009). Moreover when 5 $x10^6$ cells are pre-treated with 1 µM FTY720 for 1 hr these were shown to contain 0.1-0.2 nmol pFTY720 (Sensken et al. 2009). Notably, in the *in vitro* studies pFTY720 was used, which in this form would be unlikely taken up by cell and 100 times fewer cells ($1x10^4$) were used, which accordingly would contain <1-2pmol of pFTY720 and release even less.

In this study, splenocyte cultures were used, a mixed population of cells that include macrophages, B cells, natural killer cells and dendritic cells in addition to T cells. Analysis of these splenocyte cultures showed an approximate 60% lymphocyte population, of which more than 40% were $CD4^+$ T cells (data not shown). Notably, the cytokines analysed in our study, IL-10, IL-6, IFN-γ and TNF-α can be produced by cells other than $CD4^+$ T cells. Moreover S1PR activation has been shown to alter cytokine levels in a range of immune cells. For example in macrophages, S1PR1-specific activation attenuates the levels of TNF-α, MCP-1 and IL-6 (Potì et al. 2013b), while pFTY720 reduces levels of IL-12 and increases IL-10 in dendritic cells (Müller et al. 2005). Furthermore, S1P or pFTY720 primed dendritic cells show an altered ability to

activate T cells, with these T cells displaying less IFN-γ and more IL-4 production than is seen when activated with untreated dendritic cells (Idzko et al. 2002; Müller et al. 2005) This indicates a shift from a pro-inflammatory Th1 to an anti-inflammatory Th2 cell type (Idzko et al. 2002; Müller et al. 2005).

3.3 What is the proposed mechanism through which pFTY20 attenuates demyelination in this assay?

The internalisation of S1PRs in T cells, by pFTY720, makes them insensitive to the gradients of S1P that exist between the lymphoid organs and the peripheral circulation, which inhibits the mobility of activated T cell and prevents them from exiting lymph nodes (Mandala et al. 2002; Matloubian et al. 2004). This activity has been well characterised and explains much of the beneficial effect of this drug in MS. In addition, pFTY720 is also purported to induce a phenotypic change in some T cells that causes them to take on a Treg function (Daniel et al. 2007; Liao et al. 2007; Sehrawat & Rouse 2008; M.-G. Kim et al. 2011). Notably, however, previous studies have utilised T cell populations enriched for $CD4^{+}CD25^{-}$ cells in order to demonstrate an increase in Treg proportion induced by pFTY720 and moreover some studies have suggested that pFTY720 reduces numbers of Tregs (Wolf et al. 2009). Thus, in the current study it was decided to further examine if pFTY720 altered numbers of Tregs. For this purpose a mixed-population of splenocyte isolated from 2D2 transgenic mice was used and it was found that *in vitro* treatment of these cells with pFTY720 did not alter T cell phenotype; specifically there was no effect on CD4 and Foxp3 expressing Tregs. In contrast, the positive control, TGF-β treatment caused a large increase in this population of cells, as expected.

In addition to pFTY720 altering T cell transmigration and possible lymphocyte phenotype (although this requires further corroboration), there are suggestions that pFTY720 may also alter cytokine release from immune cells such as dendritic cells (Idzko et al. 2002; Müller et al. 2005). Moreover studies show that S1P and the selective S1PR1 modulator, SEW2871, promotes development of IL-17 expressing cells (Th17) from splenic CD4+ T cells, while pFTY720 suppressed this S1P-mediated development of Th17 cells and levels of IL-17 (Liao et al. 2007). The possibility that treatment of isolated MOG-splenocyte with pFTY720 altered the release of cytokines from these cells was examined. Firstly the levels of IFNγ were examined, given that it is a classical inflammatory cytokine that is indicated in a variety of processes in MS including Th1 cell differentiation, $CD8^{+}$ cell-induced cell death and microglial

recruitment (Panitch et al. 1987; Aloisi et al. 2000; Buntinx et al. 2002; Sepulcre et al. 2005; Murphy et al. 2010). Indeed, previous studies have shown that S1P attenuates the production of IFN-γ by splenic $CD4^+$ T cells, and this effect is likely mediated via S1PR1 (Dorsam et al. 2003). Notably, however, the direct effects of pFTY720 on IFN-γ were not examined in these reports (Dorsam et al. 2003; Liao et al. 2007). Moreover the levels of IL-6 were also tested as it has been shown to play a role in the desensitising effector T cells to regulation by Tregs in MS, as well as being elevated in the serum and CSF of MS patients when compared to healthy controls (Stelmasiak et al. 2000; Schneider et al. 2013). The effects of pFTY720 on the levels of TNF-α in MOG-reactive splenocyte were also examined, given that levels of TNF-α are elevated in CSF, serum and post-mortem in the brain lesions of MS patients, compared to healthy control and that there is evidence to suggest a link between elevated TNF-α and severity of lesions (McCoy & Tansey 2008). Interestingly, however, anti-TNF-α therapies have not been shown to be beneficial in MS and may be detrimental (van Oosten et al. 1996; Sicotte & Voskuhl 2001). In agreement with anti-inflammatory role of S1P receptors, here it was found that *in vitro* treatment with pFTY720 caused a significant concentration-dependent decrease in IFN-γ and IL-6 in activated 2D2-splenocyte, while having moderate effect on TNF-α and no effect on IL-10. These data suggest that proinflammatory cytokine release from MOG-reactive splenocyte may be involved in the demyelination process in the *in vitro* culture system. This appears consistent with the idea that pFTY720 likely causes a broad attenuation in the levels of pro-inflammatory cytokines, in agreement with this lab's previous findings (Sheridan & Dev 2012).

3.4 Conclusion

Taken as a whole, therefore, this study suggests that pFTY720 treatment of MOG-reactive splenocyte (either from MOG-immunised mice or 2D2-transgenic mice) can reduce the release of proinflammatory cytokines and can reduce the ability of these cells to induce demyelination. These results are important in the further understanding of how S1PR compounds are efficacious in multiple sclerosis and how this class of drugs may be used in neuroinflammatory and/or demyelinating illnesses.

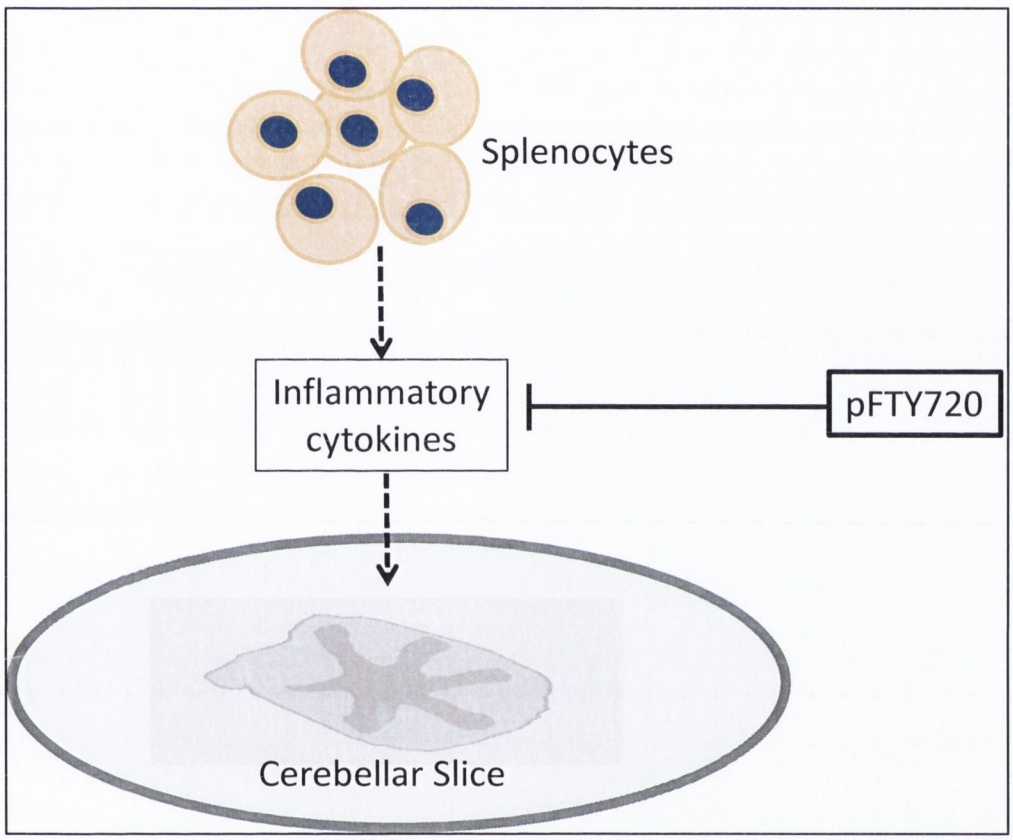

Figure 5.10 pFTY720 treatment *in vitro* reduces the release of pro-inflammatory cytokines from MOG-reactive cells and reduces demyelination.

MOG-reactive cells, reactivated in the presence of pFTY720, show a decreased release of IL6 and IFNγ. This is proposed to contribute towards the decreased demyelination seen in cerebellar slices treated with these cells when compared to cells receiving no pFTY720.

Chapter 6. Discussion

1. Opening remarks

The orphan GPCR cloned as *Endothelial Differentiation Gene-1* (EDG-1) was identified in 1990 as an abundant transcript found in human umbilical vein endothelial cells (HUVECs) (Hla & Maciag 1990). In 1996, it was proposed that S1P acted through a specific, but as yet unidentified GPCR, to induce neurite retraction in a similar manner to another GPCR-targeting lipid, lysophosphatidic acid (LPA) (Postma et al. 1996). In 1997 it was found that two other orphan GPCRs, H218 (also known as AGR16 or EDG-5) and EDG-3, were activated by S1P (An et al. 1997). The identification of these novel S1PRs, later named S1PR2 and S1PR3, was closely followed by the deorphanisation of EDG-1 in 1998, when it was reported that EDG-1 (later named S1PR1) expressed in human embryonic kidney (HEK) cells was activated by S1P (Lee et al. 1998). Over the next three years the remaining known S1PR subtypes were discovered (Yamazaki et al. 2000; Im et al. 2000). The discovery of the immunosuppressive properties of FTY720 (Adachi et al. 1995) and its consequent development as an MS therapy (Brinkmann et al. 2010) has led to much interest in the function of S1PRs. It has been shown that FTY720 is BBB permeable and, once in the CNS, it is phosphorylated to its active form by SphKs (Foster et al. 2007). In this regard, recent studies have shown that the efficacy of FTY720 as a multiple sclerosis therapy may not be due solely to the effects it exerts on T cell migration but that direct modulation of S1PRs on cells of the CNS may also be beneficial (Miron et al. 2010; Choi et al. 2011; Sheridan & Dev 2012). This current study aimed to explore the relative effects of S1PR modulation in cells of the CNS, particularly astrocytes, as well as immune cells.

2. Effects of intracellular S1PR1 modulation on ERK phosphorylation in astrocytes (Chapter 3.)

Pharmacological tools allow for the dissection of the relative contribution of each S1PR subtype to the functional effects of S1PR modulation. The tools utilised in this current study include: receptor specific agonists, such as the S1PR1 agonist AUY954; specific antagonists, such as the S1PR1 antagonist BYR185; and a novel pathway specific modulator of S1PR1 signalling, MNP301. The effects of MNP301 include inhibition of S1PR1-associated calcium signalling induced by S1P, pFTY720 and AUY954 (Healy et al. 2013) as well as inhibition of S1PR1-induced pERK in astrocytes. This MNP301-mediated inhibition of S1PR1-induced ERK phosphorylation was the focus of Chapter 3.

2.1 Summary of Results.

2.1.1 MNP301 inhibits S1PR1-activation of ERK

It was found that the phosphorylation of ERK via the activation of S1PRs by the pan-S1PR agonists, S1P and pFTY720, was partially attenuated by the S1PR1-specific antagonist BYR185. However, the phosphorylation of ERK in astrocytes treated with the specific S1PR1 agonist AUY954 was fully inhibited by BYR185. This indicates that S1PR1 activation is not solely responsible for the regulation of pERK by S1PRs and there is likely significant S1PR3 activity associated with the initiation of the ERK signalling cascade in astrocytes. Similarly, the MNP301 peptide did not attenuate levels of S1P or pFTY720-induced pERK but did inhibit the activation of ERK induced by AUY954. This data supports the hypothesis that MNP301 selectively inhibits the activation of certain pathways associated with S1PR1 agonism (**Figure 6.1**).

2.1.2 Does MNP301 inhibit pERK induced by other GPCRs?

Possible alternative explanations for the reported effects of MNP301 were explored. The possibility that MNP301 acted as a non-specific inhibitor of ERK phosphorylation was addressed. Healy et al. (2013) demonstrated that MNP301 specifically inhibited S1PR1-induced calcium signalling but not signalling through S1PR3. In Chapter 3, the effects of the peptide on signalling events initiated through unrelated GPCRs were examined. Glutamate was used to stimulate the phosphorylation of ERK as it is a ubiquitous neurotransmitter that activates ERK through its metabotropic glutamate receptors (mGluRs). It was found that MNP301 did not affect ERK activation caused by glutamate. These data, coupled with the fact that MNP301 does not affect S1PR3 induced ERK activation, as described above, indicate that the peptide acts as a specific inhibitor of S1PR1-induced ERK phosphorylation.

2.1.3 Does calcium signalling play a role in S1PR1 activation of ERK?

Intracellular calcium signalling has been described to be able to promote the activation of the ERK pathway (Chuderland & Seger 2008). In addition, Healy et al (2013) previously showed that MNP301 inhibits S1PR1-induced calcium signalling. It was therefore possible that this inhibition of calcium by MNP301 could impact on the phosphorylation of ERK and could account for the decrease in S1PR1-induced pERK seen upon addition of MNP301 in Chapter 3. However, it was found that neither EGTA, a chelator of extracellular calcium, nor dantrolene, an inhibitor of intracellular calcium release due to inhibition of ryanodine receptors, had any effect on the levels of S1PR1-activated pERK. This indicated that, in astrocytes, the activation of ERK by S1PR1 is not calcium dependent and thus the inhibition of pERK by MNP301 was not due to

inhibition of calcium signalling. These data further support the hypothesis that MNP301 is a selective inhibitor of pathways activated by specific S1PR1 agonism.

2.1.4 Where is the active site in MNP301?

Next, the location of the active site responsible for the inhibitory effects of MNP301 was sought. Three mutant peptides, named MNP302-304, were synthesised. Each contained a TAT tag and a triple alanine sequence in place of three different amino acids of the wild-type sequence of MNP301. MNP301=TAT-MSSGNVNSSS, MNP302=TAT-MSSGNVN**AAA**, MNP303=TAT-MSSG**AAA**SSS and MNP304=TAT-M**AAA**NVNSSS. These peptides, showed differing efficacy in reducing levels of pERK induced by AUY954. MNP302 reduced levels of pERK similar to those seen with the addition of MNP301. This indicated that these final three serine residues were not part of the interaction domain of MNP301. However, MNP303 and MNP304 did not reduce pERK induced through S1PR1 indicating that the active inhibitory site of the peptide is likely contained within the MSSGNVN sequence of the peptide.

2.2 Relevance and limitations of research

One or more signalling pathways may be indicated in a disease state but the agonism or antagonism of a receptor will impact on all signalling events downstream of the target receptor. Similarly, inhibition of a particular pathway through the use of a compound such as the ERK inhibitor, U0126, will inhibit activation of this cascade by all receptors and in all cells. MNP301 has been shown to selectively inhibit the activation of certain pathways, namely calcium and pERK, by S1PR1 but not activation by other S1PRs or unrelated GPCRs. In this way, it may be possible to modulate specific signalling events linked to cellular dysfunction without disrupting the function of signalling molecules that are not functioning aberrantly. Based on these data, MNP301 is a promising modulator of S1PR1 function that warrants additional research.

However, the mechanism through which MNP301 inhibits ERK phosphorylation is not yet fully understood. The peptide is designed to mimic the extreme C-terminal sequence of S1PR1 and thus is proposed to block protein-protein interactions that under normal circumstances would involve this ten amino-acid domain. When the final three serine residues of MNP301 were mutated to alanine (to create MNP302), the peptide retained an ability to inhibit ERK phosphorylation **(Chapter 3, Figure 3.9)**. However mutations at points further up the sequence (as seen in MNP303 and MNP304) removed any inhibitory effect and it was proposed that this data further refined the putative sight of interaction to a seven amino-acid sequence. Another possible explanation of this data is that the 3-D conformation of the peptide is changed

by mutating certain amino-acids within the sequence and therefore interactions between the peptide and other proteins no longer occur. Further studies are required to describe the mechanism through which MNP301 can inhibit ERK signalling induced through S1PR1.

2.3 Future studies

2.3.1 Identification of interacting proteins

While the studies described above give much insight into the activity of the S1PR1-specific pathway-selective modulator, MNP301, questions remain to be addressed. At present the mechanism through which MNP301 inhibits pERK is not known. Based on the design of the peptide as a mimic of the extreme C-terminus of the S1PR1, it is hypothesised that MNP301 prevents the interaction between a protein, or group of proteins, and the S1PR1 C-terminal thereby inhibiting the propagation of certain intracellular signals (**Figure 6.1**). Pull-down studies involving proteins of interest and the MNP301 peptide sequence would show these potential interactions and thereby help our understanding of S1PR1 function by providing a fuller explanation of proteins involved in receptor internalisation and signalling.

2.3.2 In vivo use of MNP301

The active site of MNP301 has been found to be located within the MSSGNVN sequence of the peptide. This small peptide chain could be converted into a peptomimetic that would be stable for use in an *in vivo* setting. *In vivo* studies would provide a wealth of data in regard to the functional outcomes of pathway specific modulation of S1PR1 signalling. In addition it would be possible to examine the effects of the peptomimetic on the efficacy of pFTY720 or specific S1PR1 agonists in EAE and thus establish the importance of internalisation, calcium signalling and ERK phosphorylation in astrocytes to these treatments.

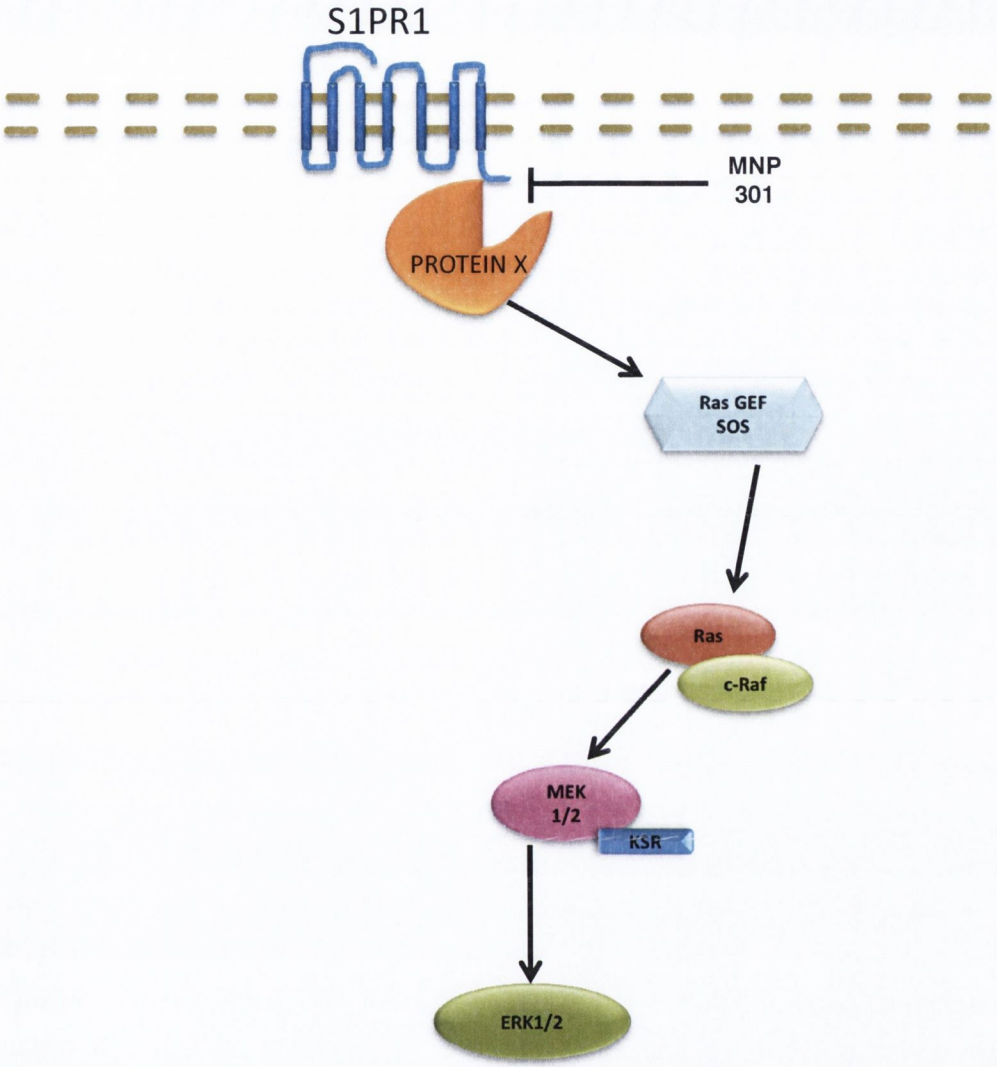

Figure 6.1 MNP301 inhibits ERK phosphorylation through an unidentified mechanism

MNP301 is designed to mimic the C-terminal of the S1PR1. Based on this design it is proposed that MNP301 inhibits the interaction between the S1PR1 C-terminal and an unidentified protein or group of protein, thereby inhibiting ERK phosphorylation.

3. Effects of intracellular S1PR1 modulation on Akt phosphorylation and migration (Chapter 4.)

Further to the effects of MNP301 on the phosphorylation of ERK in astrocytes we then examined other intracellular signalling pathways are affected by this peptide. ERK and Akt can share an upstream activator in Ras (Mendoza et al. 2011) and are both indicated in the onset and progression of tumours (De Luca et al. 2012; Chappell et al. 2011). Akt, like ERK, is a signalling molecule known to be activated downstream of growth factor receptors but also GPCRs (Lee et al. 2001). Due to the links between ERK phosphorylation and the activation of Akt as described above, the effects S1PR1 activation on Akt phosphorylation and the consequent impact of addition of MNP301 were examined.

3.1 Summary of Results

3.1.1 S1PR activation induces Akt phosphorylation that is enhanced by MNP301

S1PR associated phosphorylation of Akt has been described in numerous cell types, particularly endothelial cells (Lee et al. 2001), fibroblasts (Baudhuin et al. 2004) and T cells (Liu et al. 2009) but is not well described in astrocytes. The current study demonstrated that S1PR1 activation by AUY954 induces phosphorylation of Akt that is both time and dose-dependent. This phosphorylation of Akt via S1PR1 activation is reversed by the addition of the S1PR1 antagonist BYR185. It was hypothesised that the addition of MNP301 to astrocytes would inhibit the S1PR1-induced phosphorylation of Akt similar to that seen with ERK. However, it was found that MNP301 potentiated the phosphorylation of Akt by AUY954 to levels greater than those seen with AUY954 alone.

3.1.2 What is the mechanism whereby MNP301 enhances pAkt?

The underlying mechanism regulating this enhanced phosphorylation of Akt was unclear but it was thought that it may be linked to the inhibition of pERK as described in chapter 3. Cross-talk between the Akt and ERK pathways has been recognised in many cell types (Irie et al. 2005; Serra et al. 2011; Normanno et al. 2006; Mendoza et al. 2011) and was thought to be involved in this increased levels of pAkt seen here in astrocytes. To investigate this possibility, U0126 was used to inhibit the activation of MEK1/2, kinases that directly phosphorylate and hence activate ERK. It was found that U0126 enhanced and prolonged the phosphorylation of Akt induced by AUY954. Furthermore, treatment of cells with U0126 alone induced phosphorylation of Akt. Together these data indicated that inhibition of pERK by U0126 could induce Akt

phosphorylation and potentiated pAkt induced by S1PR1 activation (**Figure 6.2**). In this current study, it was hypothesised that MNP301 and U0126 increase Akt phosphorylation by inhibiting ERK activation and thus remove a negative feedback signal that originates from the ERK pathway thereby reducing pAkt. This hypothesis is in agreement with Mendoza et al. (2011) who describe that ERK can inhibit the activation of Grb2-associated binding protein (GAB), upstream of Akt, thereby reducing pAkt levels.

3.1.2 The effect of S1PR1 activation of Akt on astrocyte migration

The functional effects of ERK/Akt cross-talk were examined in an astrocyte migration assay. S1PR1 activation has previously been shown to induce significant levels of astrocyte migration when compared to control (Mullershausen et al. 2007). In this current study we established that pFTY720 (10nM) and AUY954 (100nM) cause significant migration of astrocytes that is fully inhibited by the S1PR1 specific antagonist BYR185, indicating that astrocyte migration is specifically regulated by S1PR1 activation. Akt phosphorylation has been linked to migration of cells such as endothelial (Irie et al. 2005) and epithelial cells (Lee et al. 2001). In order to ascertain whether Akt phosphorylation is required for the S1PR1 induced migration of astrocytes; cells were treated with the specific Akt inhibitor, MK2206, prior to treatment with pFTY720 or AUY954. MK2206 significantly restricted astrocyte migration induced by both S1PR agonists. This demonstrates that Akt phosphorylation regulates astrocyte migration stimulated through S1PR1.

3.1.2 The effect of pERK inhibition on astrocyte migration

The effect of the of the MEK inhibitor U0126 on astrocyte migration was then assessed. U0126 stimulated astrocyte migration to levels similar to those seen with both pFTY720 and AUY954. MNP301 also induced significant migration of astrocytes. It is noteworthy that despite U0126 and MNP301 potentiating the phosphorylation of Akt induced through S1PR1, as described above, co-treatment of U0126 or MNP301 and pFTY720 or AUY954 did not increase levels of astrocyte migration when compared to individual treatments. It is possible that the enhanced levels of pAkt seen upon co-treatment are not sufficient to induce a functional elevation in migration. Alternatively, the levels of migration seen with both single and co-treatment may represent a maximal response.

3.1.2 Do U0126 and MNP301 promote migration due to pAkt?

Based on these data it is likely that impairment of the activation of ERK, and consequent increase in Akt phosphorylation, due to U0126 and MNP301, contributes to astrocyte migration. To confirm this hypothesis, astrocytes were treated with U0126 or MNP301 in the presence of the Akt inhibitor MK2206. It was shown that MK2206, negated astrocyte migration induced by MNP301 while it also attenuated U0126-related migration. These results indicate that MNP301 and U0126 promote astrocyte migration via inhibition of pERK and the associated recruitment of pAkt. U0126 is a pan-inhibitor of ERK phosphorylation whereas MNP301 only inhibits pERK due to S1PR1 activation. Therefore, why does MNP301 promote migration in an Akt dependent manner in the absence of S1PR1 activation? An explanation for this may be constitutive S1PR1 activity, which has previously been described in HEK cells (Waters et al. 2006). It may be the case that constitutive S1PR1 activity induces low level or persistent ERK phosphorylation that is inhibited by MNP301, thus increasing pAKT and increasing astrocyte migration. If this is the case it would be expected that MNP301 would reduce the levels of pERK when compared to basal control levels. The data displayed in Chapter 3 (**Figures 3.4** and **3.5**) does not show that MNP301 significantly reduce basal pERK levels, however, this may be explained by the fact that the basal level of pERK is low. A small decrease in basal pERK levels induced by MNP301 may not be obvious in a Western blot but may have a significant functional effect over a 24 hour treatment as seen in the migration assay.

Additionally, the fact that BYR185 reduces migration compared to that seen in control samples indicates that the astrocytes release S1P *in vitro* which may act through S1PR1 to contribute to a basal level of migration. Modulation of this endogenous signalling mechanism by MNP301 may contribute to the significant increase in migration seen upon addition of the peptide in the absence of an exogenous agonist. However, while possible, this study has not examined such mechanisms.

Collectively, these results indicate that cross-talk between Akt and ERK, whereby inhibition of ERK allows for an increase in the levels of pAkt, is a mechanism that exists in astrocytes (**Figure 4.2**). Inhibition of pERK by U0126 or MNP301 enhances and prolongs the phosphorylation of Akt seen upon stimulation of astrocytes with S1PR1 agonist AUY954. Functionally, this cross-talk can be utilised to promote astrocyte migration to levels similar to those seen with S1PR stimulation (**Figure 4.2**).

3.2 Relevance and limitations of research

This chapter demonstrates that agonism of S1PR1 by AUY954 induces the phosphorylation of Akt in astrocytes, a mechanism that has not been well described until now. The enhancement of this Akt signal by MNP301 and the demonstration that this effect is likely mediated by cross-talk with the ERK pathway is also novel. The phosphorylation of Akt by SEW2871-induced S1PR1 activation has been linked to OPC survival (Miron, et al. 2008; Coelho et al. 2007). It may be the case that MNP301 through regulation of ERK signalling and an increase in the levels of pAkt, can enhance OPC survival. If so, a peptomimetic compound that replicates the configuration and effects of the active site of MNP301 (MSSGNVN, as described above), may have beneficial effects on myelination state during S1PR targeted treatment.

In addition, Mullershausen et al (2007) demonstrated that astrocyte migration can be enhanced by S1PR1 activation. This current study has described that the phosphorylation of Akt is necessary for S1PR1-associated migration, a mechanism that was previously undescribed. While studies have shown that both Akt and S1PRs can be involved in cellular migration (Irie et al. 2005), it has not been shown, to our knowledge, that activation of Akt via S1PR1 promotes astrocyte migration. Astrocytes release growth factors and act to maintain the structure of the BBB. Therefore increased astrocyte motility could be beneficial in inflammatory or demyelinating conditions by allowing these effects to be more widespread.

As mentioned previously, BYR185 impairs astrocyte migration when compared to untreated control values, indicating that signalling through S1PR1 contributes to the basal level of migration seen in this study. It has not been established whether or not these astrocytes release S1P *in vitro* and whether autocrine/paracrine signalling induced by the endogenous ligand or constitutive activation of receptors contributes to astrocyte migration. Modulation of these proposed signalling events by MNP301 could explain how the peptide induces migration in the absence of an exogenous ligand for S1PR1.

3.3 Future Studies

In this chapter, MNP301 has been shown to increase levels of pAkt upon S1PR1 activation as well as promote astrocyte migration. This migration should be examined in a cerebellar slice assay, post demyelinating insult or other injury. In this way, it may

be possible to identify whether increased astrocyte migration, mediated through S1PR1, is beneficial or detrimental in demyelinating or neurodegenerative disorders. Benefits would likely be mediated by increase astrocyte migration to the injury site and a release of growth factors. Detrimental effects associated with increased migration would include increased astrogliosis, creating a glial scar and inhibiting repair.

The data gathered in chapter 4 has shown that MNP301 potentiates Akt phosphorylation due to S1PR1 activation. The phosphorylation of Akt via S1PR1 has been shown to increase OPC survival (Miron, et al. 2008; Jung et al. 2007; Jaillard et al. 2005; Coelho et al. 2007) and would be worthwhile to establish whether MNP301 enhances S1PR1-induced pAkt in OPCs as well as astrocytes. Further, if pAkt is enhanced by MNP301 in OPCs, the consequent effect on OPC survival should be assessed. Furthermore, OPC migration is thought to contribute to the remyelination process (Fancy et al. 2004) and therefore the ability of S1PR1 agonism and the pathway modulator MNP301 to promote migration in OPCs and other CNS cell types should be examined. OPCs are difficult to maintain in a single-cell culture (Pedraza et al. 2008; Najm et al. 2011), therefore the potential effects of MNP301 on OPC survival and migration could be examined in an organotypic cerebellar slice assay in a similar manner to the proposed study involving astrocyte migration.

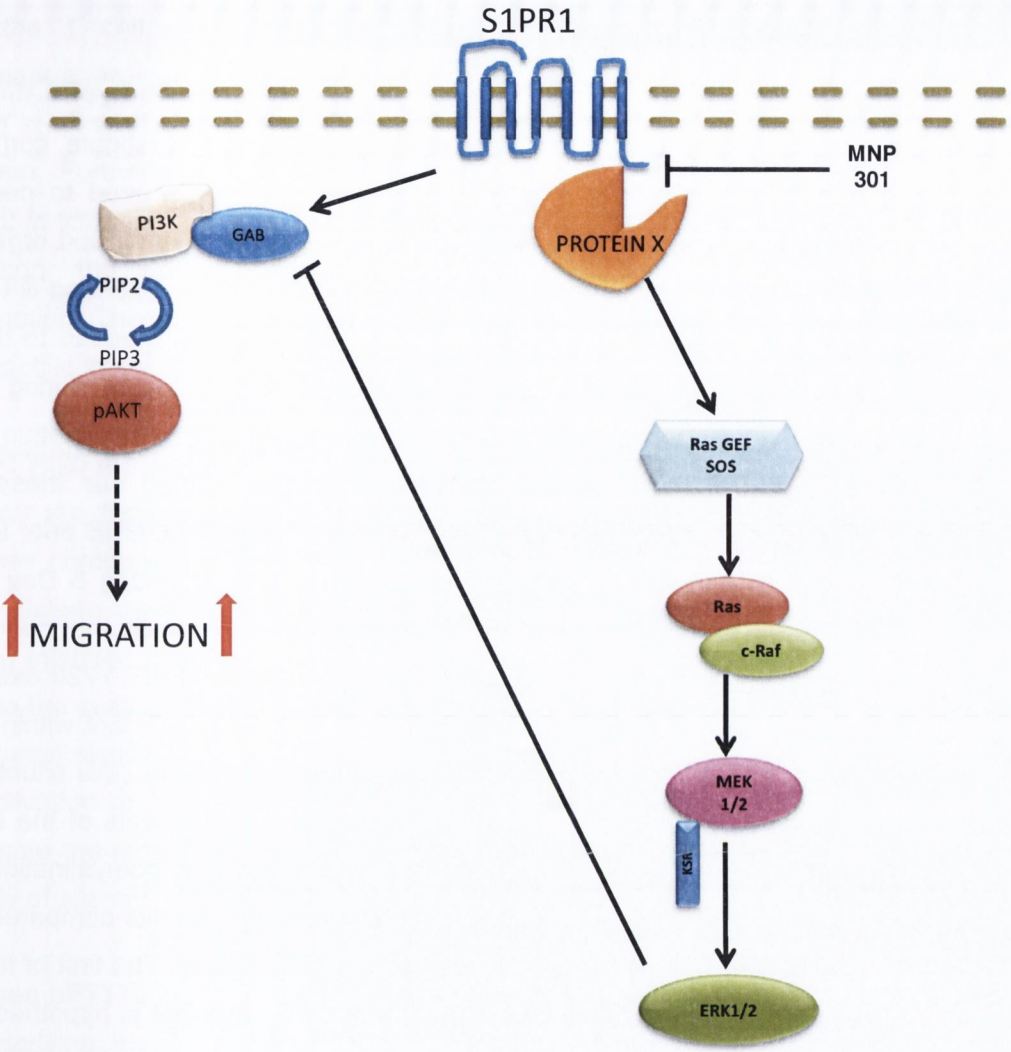

Figure 6.2 MNP301 regulates cross-talk between pERK and pAkt

MNP301 inhibits the interaction between the S1PR1 C-terminal and an unidentified protein or group of protein, thereby inhibiting ERK phosphorylation. This, in turn removes an inhibitory signal that normally regulates pAkt, thereby increasing Akt phosphorylation. The phosphorylation of Akt has been shown to regulate astrocyte migration in Chapter 4

4 Mechanism of inhibition of immune-induced demyelination in organotypic cultures (Chapter 5)

4.1 pFTY720 enhances myelination state in *in vitro* demyelination assays

Organotypic brain slice cultures provide a means to investigate complex cellular processes in a physiologically relevant model without the need to perform *in vivo* studies. Previous studies in this lab, as well as others, have utilised organotypic slice cultures to model demyelination *in vitro* (Miron et al. 2010; Sheridan & Dev 2012). In general, a demyelinating agent such as LPC or cuprizone is added to the slice for a defined period of time. Compounds are added to the culture during or after this demyelination phase, depending on whether it is reduced demyelination or increased remyelination that is being investigated. It has been noted that these organotypic cultures contain resident immune cells that have entered the CNS prior to preparation of the culture (Ling et al. 2008; Prodinger et al. 2011; Sheridan & Dev 2012). While pFTY720 undoubtedly exerts direct effects on glia and neurons that may contribute to the compound's therapeutic effects it is also possible that pFTY720 exerts beneficial effects by acting on these immune cells that are already resident within the slice. To identify if the activity of pFTY720, directly acting on immune cells situated within the brain may inhibit demyelination, while controlling for the effects of the compound on glia and neurons, an immune cell/brain slice co-culture demyelination assay was developed. We deemed that this assay had two main benefits compared to the LPC-induced demyelination slice assay we had used previously. The first of these was that demyelination would be induced in a manner similar to what is hypothesised to occur in autoimmune demyelinating disorders such as MS. Secondly, using this assay it was possible to assess what contribution the direct action of pFTY720 may have on peripheral immune cells that have passed into the CNS.

4.2 Summary of results

4.2.1 pFTY720 inhibits demyelination induced by MOG-reactive immune cells

It was found that, when spleen cells taken from MOG-immunised mice were restimulated *in vitro* and added directly to an organotypic cerebellar slice, demyelination occurred that approximated the levels of demyelination seen with LPC treatment (**Figure 6.3**). This was a validation of the assay and enabled an investigation into the effects of pFTY720 on these splenocytes and their consequent ability to induce demyelination. *In vivo* treatment with FTY720 during the MOG-immunisation protocol inhibited splenocyte-induced demyelination (**Figure 6.3**). This effect may have been due to a change in the relative proportions of T cell subsets within the spleen

resulting from FTY720 treatment. It has been reported that central memory T cells (Tcm), the T cell subtype that are suspected to cross the BBB and induce demyelination, are preferentially retained within lymph nodes upon FTY720 treatment (Mehling et al. 2008; Brinkmann 2009). These cells would therefore be lacking within the spleen. Furthermore, it was found that MOG-reactive cells, in the presence of pFTY720 *in vitro,* no longer induced significant levels of demyelination (**Figure 6.3**). In comparison, the same cells, reactivated without pFTY720, induced significant demyelination. These data indicated that pFTY720 *in vitro,* caused a change in these MOG-reactive immune cells thus functionally impairing them.

4.2.2 pFTY720 inhibits demyelination induced by 2D2-splenocytes

2D2 mice are transgenic animals that express a T cell receptor that specifically recognises components of MOG and are activated in the presence of the MOG_{35-55} peptide. Spleen cells were isolated from these mice to support the hypothesis that pFTY720 inhibited the ability of MOG-specific immune cells to induce demyelination. Similar to the cells isolated from the spleens of the MOG-immunised mice, these 2D2 cells induced significant demyelination in the organotypic co-culture assay. In addition, the reactivation of these cells in the presence of pFTY720 impaired their ability to demyelinate the slice culture. This further confirmed the ability of pFTY720 to reduce the ability of specific MOG-reactive immune cells to induce demyelination.

4.2.3 Does pFTY720 alter T cell phenotype within a mixed splenocyte culture?

The underlying mechanism whereby pFTY720 inhibited demyelination induced by MOG-reactive splenocytes was examined. Firstly, changes to the T cell phenotype within this mixed splenocyte culture were explored. It has been reported that Treg populations may be augmented by pFTY720, both *in vivo* and *in vitro* (Daniel et al. 2007; Sehrawat & Rouse 2008; Kim et al. 2011; Sun et al. 2011). Based on these data, the relative expression of CD4, CD25 and Foxp3, three markers of the Treg phenotype, in pFTY720 treated and non-treated activated 2D2 cells was examined in this current study. No difference in the expression of CD4, CD25 or Foxp3 was seen in non-treated and pFTY720-treated activated 2D2-splenocytes. In order to validate the assay and ensure that the culture conditions were conducive to the expansion of a Treg population, cells were also treated with TGF-β. These TGF-β treated splenocytes displayed up to a 20-fold increase in the population of Tregs when compared to pFTY720 treated and control samples showing that the culture conditions were suitable for Treg expansion. These data show that pFTY720 does not increase the relative proportion of Tregs in a mixed 2D2-splenocyte culture. This does not agree

with previously published findings (Sehrawat & Rouse 2008; Kim et al. 2011; Sun et al. 2011), in which Tregs were seen to proliferate within both purified T cell populations and splenocyte cultures. Therefore, in this assay system, the inhibition of demyelination ability seen in 2D2-splenocytes is not likely due to increased regulatory ability of T cells.

4.2.4 Does pFTY720 alter cytokine release by 2D2-splenocytes?

While no change was seen in the CD4, CD25 or Foxp3 expression, it was possible that the cytokine release by these 2D2-splenocytes could be altered by pFTY720. In this regard, the relative levels of IL-10, TNF-α, IL-6 and IFN-γ were measured via ELISA. No change in the level of the anti-inflammatory cytokine IL-10 was seen. In addition, while there was a trend towards a decrease in the levels of TNF-α in the presence of increasing levels of pFTY720, no significant reduction was seen. However, there was a significant dose-dependent decrease in the levels of both IL-6 and IFN-γ associated with the addition of pFTY720. Based on this cytokine data, it appears that activation of MOG-reactive 2D2 cells in the presence of pFTY720 reduces the levels of selected pro-inflammatory cytokines while leaving the levels of the anti-inflammatory cytokines examined unaffected. Sheridan et al (2012) identified a change in chemokine expression as a possible mechanism through which pFTY720 could inhibit LPC-induced demyelination in cerebellar slice cultures. In this current study a reduction in the inflammatory cytokine release by MOG-reactive immune cells due to pFTY720 treatment is likely a mechanism involved in the decreased demyelination seen.

4.3 Relevance and limitations of research

FTY720 causes the retention of lymphocytes within the lymphoid organs, thus reducing infiltration of auto-reactive T cells into the brain and consequent development of lesions. However, FTY720 will only be administered after the development of lesions, meaning that a population of immune cells has already crossed into the brain and may still reside there. Furthermore, numerous studies involving organotypic brain slices, have shown that peripheral immune cells are present in the brain of healthy animals (Ling et al. 2008; Prodinger et al. 2011; Sheridan & Dev 2012). The effect of pFTY720 on these brain-resident peripheral immune cells is not known. In Chapter 5, these brain-resident peripheral immune cells have been modelled in a MOG-reactive splenocyte/organotypic cerebellar slice co-culture assay. These data indicate that the protective effects that pFTY720 has been seen to have on myelination state in chemically-induced demyelination (Miron et al. 2010; Sheridan & Dev 2012) could be partially mediated by direct effects of S1PR modulation on CNS residing immune cells.

There is much evidence to suggest that direct effects of pFTY720 on glia can be beneficial in EAE and MS. For instance, oligodendrocyte survival and OPC differentiation and migration are regulated by pFTY720 (Jung et al. 2007) while S1PR1 expression on astrocytes has been shown to be required for pFTY720 efficacy in EAE (Choi et al. 2011). Therefore, rather than precluding a beneficial role of direct pFTY720 activity on glia in MS, the data in Chapter 5 describes an additional mechanism whereby pFTY720 may exert beneficial effects in the CNS by acting on peripheral immune cells residing therein.

4.4 Future Studies

4.4.1 Examining the effects of pFTY720 on individual immune cell types

As stated previously, the MOG-reactive splenocytes utilised in chapter 5 are a mixed cell population. A mixed culture was used in order to closer mimic the immune system effects seen in MS, where numerous cell types cross the BBB and enter the CNS. T cells are deemed to be the main proponents of demyelination but other immune cells also play a role. Dendritic cells and macrophages act as antigen presenting cells (APCs) to facilitate T cell reactivation, while also releasing inflammatory cytokines (Banchereau & Steinman 1998; Schindler et al. 2001; Brinkmann et al. 2010). B cells mature into plasma cells and release auto-antibody which may also contribute to demyelination (Hartung & Kieseier 2010). The effects of pFTY720 on these non-T cell splenocytes may contribute to the reduction in MOG-reactive splenocyte induced demyelination seen in this chapter. For instance, it has been shown that pFTY720 acting on dendritic cells may alter cytokine release and promote an anti-inflammatory Th2 rather than pro-inflammatory Th1 cell type when these dendritic cells act as APCs (Idzko et al. 2002; Müller et al. 2005). This is a possible mechanism whereby pFTY720 may reduce the pro-inflammatory cytokine release in a mixed splenocyte culture, as seen in chapter 5. In order to examine the effects of pFTY720 on individual cell types, purified cultures of each could be set up and treated with pFTY720. By washing out the pFTY720 and recombining one or more cell type cultures the functional effects of pFTY720 on the interactions of these different cells could be examined. However, the proposed experiment may not replicate the reality of pFTY720 activity *in vivo* as the compound would not selectively modulate S1PRs on one cell type while leaving others unaffected.

4.4.2 Examining the effects of pFTY720 on different effector T cell subsets

IFN-γ is typically released by Th1 cells (Murphy et al. 2010) and a reduction in the levels of this cytokine could be indicative of a reduction in the presence of this T cell subset. The relative expression of Th2 cells may be unaffected as no change in IL-10, a cytokine released by Th2 cells (Ozenci et al. 1999), due to pFTY720 has been shown in this current chapter. IL-17 and the possibility of changes in Th17 cell populations has not been examined in this study, although published data does link S1P receptors to the regulation of Th17 cell populations (Liao et al. 2007). Conclusions on the relative abundance of these $CD4^+$ T helper cells cannot be drawn based on this study due the lack of IL-17 release data and to the number of cells other than effector T cells that can release IFN-γ and IL-10 (Ozenci et al. 1999; Schindler et al. 2001). In order to ascertain the relative populations of effector T cell subsets in the MOG-reactive splenocyte culture used in this study, FACs analysis could be utilised. A protein transport inhibitor, brefeldin A (Golgiplugg), would prevent release of cytokines without inhibiting their production thus leaving them accumulated within the cell. In conjunction with a cell surface marker such as CD4 to identify broader T cell subsets, these accumulated cytokines could act as intracellular markers and indicate the relative abundance of effector T cell subsets in response to pFTY720 treatment.

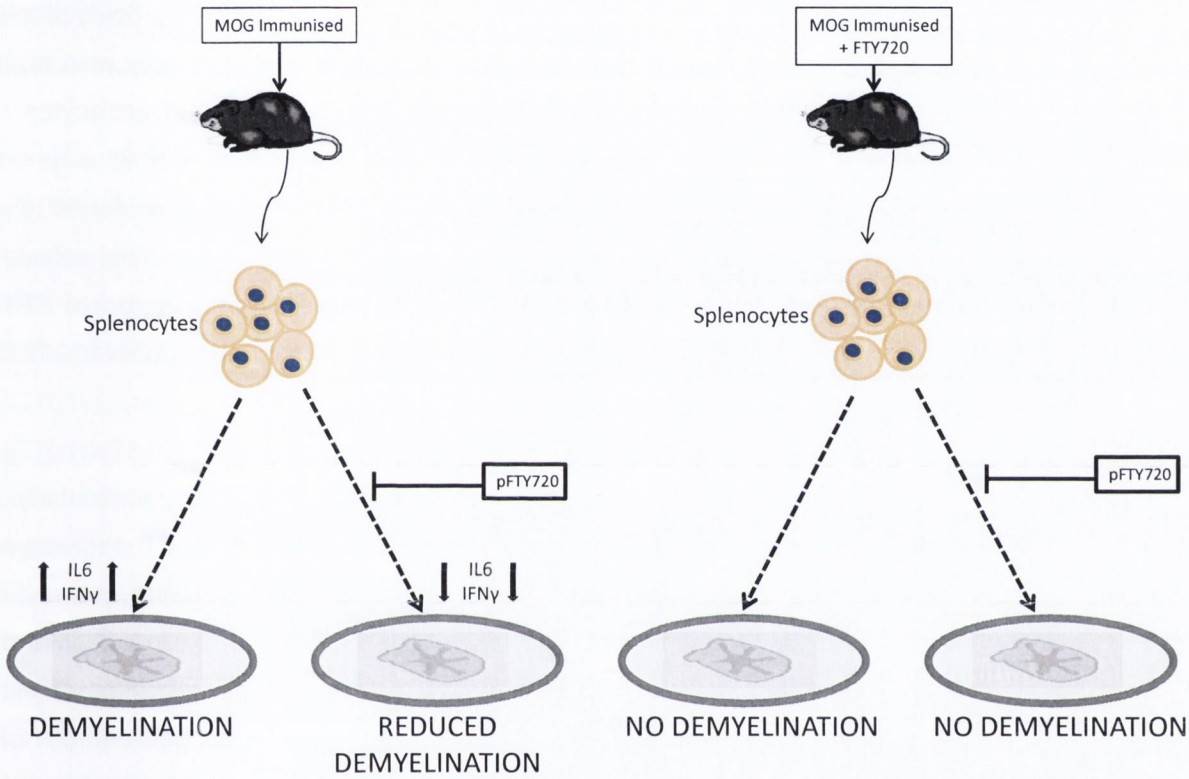

Figure 6.3 pFTY720 reduces MOG-reactive splenocyte induced demyelination

MOG-reactive splenocytes isolated from MOG-immunised or 2D2 mice induce significant demyelination of cerebellar slices *in vitro*. This demyelination is inhibited by the addition of pFTY720 *in vivo* and/or *in vitro*. It is proposed that a reduction in inflammatory cytokine release contributes to this effect.

5 Concluding remarks

In an *in vitro* demyelination model, the activation of S1PRs decreases demyelination and increases remyelination (Miron et al. 2010; Sheridan & Dev 2012). This protection from demyelination and enhanced repair is also linked to a change in the cytokine, reactive oxygen species and caspase profile (Miron et al. 2010; Jackson et al. 2011; Sheridan & Dev 2012). Thus, it is possible that pFTY720 decreases demyelination and enhances remyelination by creating a pro-survival, anti-inflammatory environment for oligodendrocytes. Furthermore, specific knockdown of S1PR1 in astrocytes renders FTY720 ineffective in EAE, suggesting an important role for S1PR1 expressed on these glia in MS therapy (Choi et al. 2011). As such there is much evidence to suggest that direct CNS effects of FTY720 contribute to the overall beneficial effects of the compound. When combined with established data regarding the impact of FTY720 on the trafficking of lymphocytes, it seems that this S1PR agonist can exert effects both within the peripheral immune system and the CNS.

This current study has demonstrated that activation of S1PRs, particularly S1PR1 activates intracellular signalling pathways that can be altered by the pathway specific, S1PR1-selective modulator, MNP301 **(Chapter 3)**. This signalling modulation was seen to promote astrocyte migration, an effect of directly targeting S1PRs on CNS derived cells **(Chapter 4)**. The ability of MNP301 to promote migration of astrocytes was attributed to regulation of ERK **(Chapter 3)** and cross-talk between the ERK and Akt signalling pathways **(Chapter 4)**, an event not previously described in astrocytes (**Figure 6.4**).

In addition, MOG-reactive immune cells treated with pFTY720 were shown to lose the ability to induce demyelination in a cerebellar slice culture model **(Chapter 5)**. It is proposed that the reduction in the inflammatory cytokines released by these cells, attributed to the addition of pFTY720, contributes to this decreased demyelination (**Figure 6.4**). These data have relevance to the population of peripheral immune cells that may have already crossed the BBB and are resident within the CNS of the MS patient prior to the initiation of pFTY720 treatment. A reduction in the inflammatory products released by these brain-resident immune cells would contribute to the benefits of FTY720 therapy already described.

The impact of the modulation of S1PR1 signalling with regard to ERK/Akt crosstalk in astrocytes and the associated increase in astrocyte migration was not examined in a whole slice culture system. It is possible that the increased migration induced by MNP301 may have beneficial effects in a demyelination model due to an earlier presence of astrocytes as a result of their increased motility. Conversely, such an effect may inhibit repair of injury due to increased gliosis. Studies to investigate these possibilities will provide a better understanding of the importance of astrocyte migration to the repair of demyelination.

The efficacy of FTY720 in the treatment of EAE and MS has led to increased interest in S1PR as potential drug targets in a variety of other auto-immune and inflammatory disorders. There are currently clinical trials underway investigating the use of novel S1PR agonists or modulators in the treatment of RRMS (Clinical trial identifiers: NCT00879658, NCT01185821, NCT01093326, NCT01093326), SPMS (NCT01665144), hepatic impairments (NCT01565902), and ulcerative colitis (NCT01647516). FTY720 is being investigated for efficacy in conditions as diverse as acute non-infectious intermediate posterior and pan-uveitis (NCT01791192), amyotrophic lateral sclerosis (ALS) (NCT01786174), schizophrenia (NCT01779700), acute demyelinating optic neuritis (NCT01757691), RRMS with depression in combination with antidepressants (NCT01436643), chronic inflammatory demyelinating polyradiculoneuropathy (NCT01625182) and kidney transplant (NCT00099801). The relative contribution of the immune and CNS effects of S1PR modulation to disease therapy is currently being debated. However, as can be seen from the variety of diseases now being investigated in relation to S1PR therapies, much benefit to patients may result from a continued effort to identify and explain the mechanism underlying the activity of these receptors.

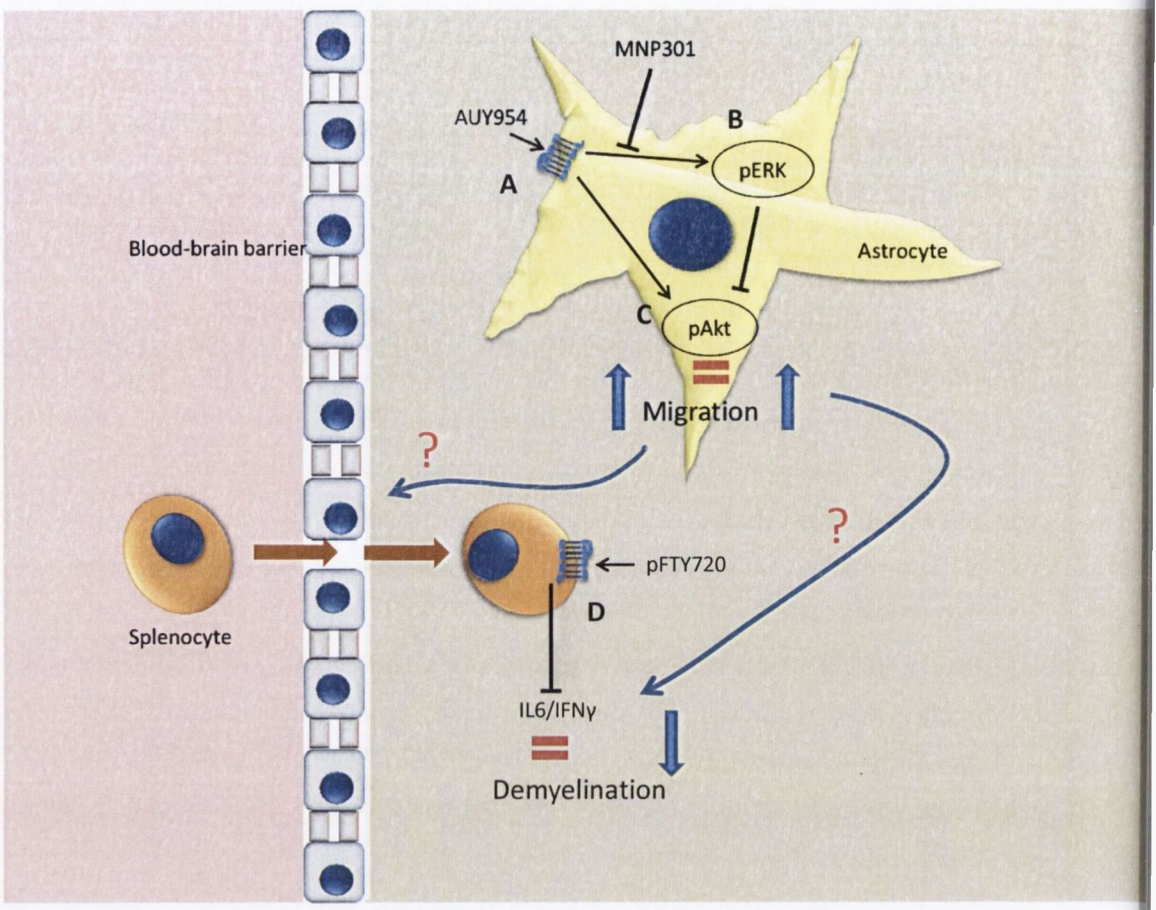

Figure 6.4 Summary of findings

(A) Activation of S1PR1 on astrocytes induces ERK activation that is inhibited by MNP301. **(B)** MNP301 also regulates crosstalk between pERK and pAKT, increasing levels of the latter. **(C)** This is proposed to be the mechanism through which MNP301 can promote astrocyte migration. The beneficial effects of this migration may be beneficial to remyelination and structural repair of the BBB although this has not been established. **(D)** pFTY720 has also been shown to decrease immune-cell mediated demyelination, possibly by reducing levels of inflammatory cytokines.

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