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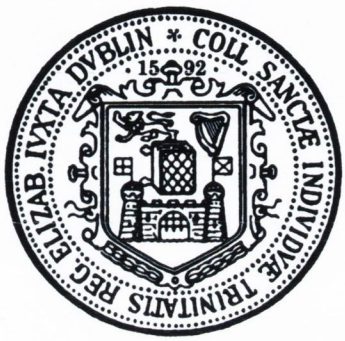
THE REGULATION OF SPHINGOSINE-1-PHOSPHATE 1 RECEPTOR SIGNALLING AND TRAFFICKING IN ASTROCYTES: IMPLICATIONS FOR MULTIPLE SCLEROSIS.



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

Luke M. Healy

September 2012

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Declaration

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Abstract

Multiple Sclerosis (MS) is a chronic, inflammatory and autoimmune disease of the central nervous system (CNS). Fingolimod (FTY720-P) is an immunomodulatory drug recently granted FDA approval for the treatment of remitting relapsing MS. This compound is a pro-drug and an analogue of sphingosine-1-phosphate (S1P), an important lipid mediator that is implicated in a wide range of biological processes. The clinical benefits of FTY720-P have been attributed to its modulation of a group of G-protein coupled receptors (GPCRs) which naturally bind S1P. Internalisation of these S1P receptors by binding of FTY720-P limits entry of autoreactive T-cells into brain tissue. This reduces the occurrence of localised swelling and subsequent damage to the brain parenchyma. Activation of S1P receptors plays a role in cellular migration, survival and differentiation in a wide range of cells. S1P1 receptors are ubiquitously expressed throughout the brain in neurons and glia. Furthermore FTY720-P crosses the blood brain barrier and localises to the CNS white matter, particularly the myelin sheath. We hypothesise that efficacy of FTY720-P in the treatment of MS is not solely due to its effect on the peripheral immune system but also due to a direct effect on the cells of the CNS. We are particularly interested in FTY720-P's action on astrocytes. These cells play a significant role in the proper functioning of the brain. We have shown that FTY720-P promotes astrocyte migration through modulation of S1P1 receptors. The aim of this project is to study the effects of FTY720-P on the internalisation and trafficking of the S1P1 receptor in astrocytes, with the hope of better understanding its mechanism of action in the context of MS. We hope to investigate this by using primary cell cultures in conjunction with immunocytochemistry and biochemical studies.

Author Keywords: G-protein coupled receptors, multiple sclerosis, sphingosine-1-phosphate, FTY720-P, astrocytes, and receptor internalisation.

Abbreviations

Ab	antibody
APC	antigen-presenting cell
ATP	adenosine triphosphate
BBB	blood brain barrier
Ca^{2+}	calcium
$_{i}Ca^{2+}$	intracellular calcium
CHAK	CC-chemokine-activated killer
CNS	central nervous system
CD62L	L-selectin
CSF	cerebrospinal fluid
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalitis
ExcL	extracellular loop
Edg	endothelial differentiation genes
ER	endoplasmic reticulum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
Fsk	forskolin
GA	glatiramer acetate
GABA	γ-aminobutyric acid
Gd	gadolinium
GPCR	g-protein coupled receptor
GRKs	g-protein-coupled receptor kinases
GWA	genome wide association
GlyCAM-1	glycosylation dependent cell adhesion molecule-1
HDL	high density lipoprotein
I_{CRAC}	calcium release activated calcium current
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol (1,4,5) triphosphate
LIF	leukemia inhibitory factor
LIX	LPS induced CXC chemokine
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NO	nitric oxide
NT-3	neurotrophin-3
OPC	oligodendrocyte progenitor cell
PDZ	PSD-95, Dlg, ZO-1 homology domain
PKC	protein kinase C
PLP	proteolipid protein
PNS	peripheral nervous system
PPMS	primary progressive multiple sclerosis
PTD	protein transduction domain
RANTES	regulated upon activation, normal T- cell expressed possibly secreted

RNS	reactive nitrogen species
ROS	reactive oxygen species
RRMS	relapsing remitting multiple sclerosis
SNPs	single nucleotide polymorphisms
SOCE	store operated calcium entry
S1P	sphingosine-1-phosphate
S1P1	sphingosine-1-phosphate receptor 1
SphK	sphingosine kinase
SPMS	secondary progressive multiple sclerosis
TAT	trans-activating transcriptional activator
T_{CM}	central memory T-cell
T_{EM}	effector memory T-cell
TGN	trans-Golgi network
TNF-α	tumour necrosis factor-α
VCAM	vascular cell adhesion molecule
VLA	very late antigen

Chapter 1. Introduction.

1.1 Multiple Sclerosis

1.1.1 The Disseminating Sclerosis.

The complexity and diversity in function of the nervous system is dependent on interconnections between neurons. The basic functionality of the brain depends on the ability of neurons to receive, process and propagate an electrical impulse. This feature of a neuron is, in part, facilitated by the myelin sheath which is a fatty material that surrounds and insulates the axons of neurons. This sheath allows for the proper conduction of action potentials down an axon. Myelin is the outgrowth of glial cells, oligodendrocytes in the central nervous system (CNS) (Compston and Coles, 2002). Multiple Sclerosis (MS) was first scientifically described, named and documented by the "founder of modern neurology" Dr. Jean-Martin Charcot in 1868. MS is a neurodegenerative inflammatory disease of the CNS with a distinct autoimmune aspect. In young adults MS is the most common non-traumatic cause of neurological disability. Although much is known about the disease process the etiology of MS is unknown and, while it is not considered a hereditary disease, it is thought the disease may be caused by a combination of genetic factors and environmental or infectious triggers (Pilz et al., 2008). The disease manifests itself as acute focal inflammation, axonal loss caused by demyelination, with limited remyelination. MS refers to the scars (scleroses) or disseminated white matter lesions that are the pathological hallmark of this debilitating disease. Although these lesions or plaques can occur throughout the white matter the most commonly affected areas are the periventricular regions, brainstem, optic nerve and the spinal cord (Noseworthy et al., 2000). It is also known that the blood brain barrier (BBB) is disrupted in MS; however, debate remains as to which occurs first, demyelination or inflammation (Blakemore, 2008). Patients present to their general practitioner with a clinically isolated syndrome which is defined as an individual's first neurological episode caused by inflammation or demyelination of the nervous tissue. A clinically isolated syndrome usually presents as optic neuritis caused by an inflammatory lesion along the optic nerve's axonal tract, a remission period usually follows (Thrower, 2007). There are four main forms of MS, namely relapsing remitting, primary progressive, secondary progressive and progressive relapsing MS (**Fig.** 1.1) (Frohman et al., 2005). The major form of MS is relapsing remitting, from which approximately 90% of patients suffer. MS patients can experience almost any neurological symptom. However the more common symptoms are vision loss, ataxia, tremors, muscle spasticity, paralysis and cognitive impairment (ranging from short term memory loss to depression). One of the most disabling symptoms is loss of gait control caused when a lesion occurs in the motor cortex, leading to patients becoming wheelchair bound (Confavreux et al., 2000).

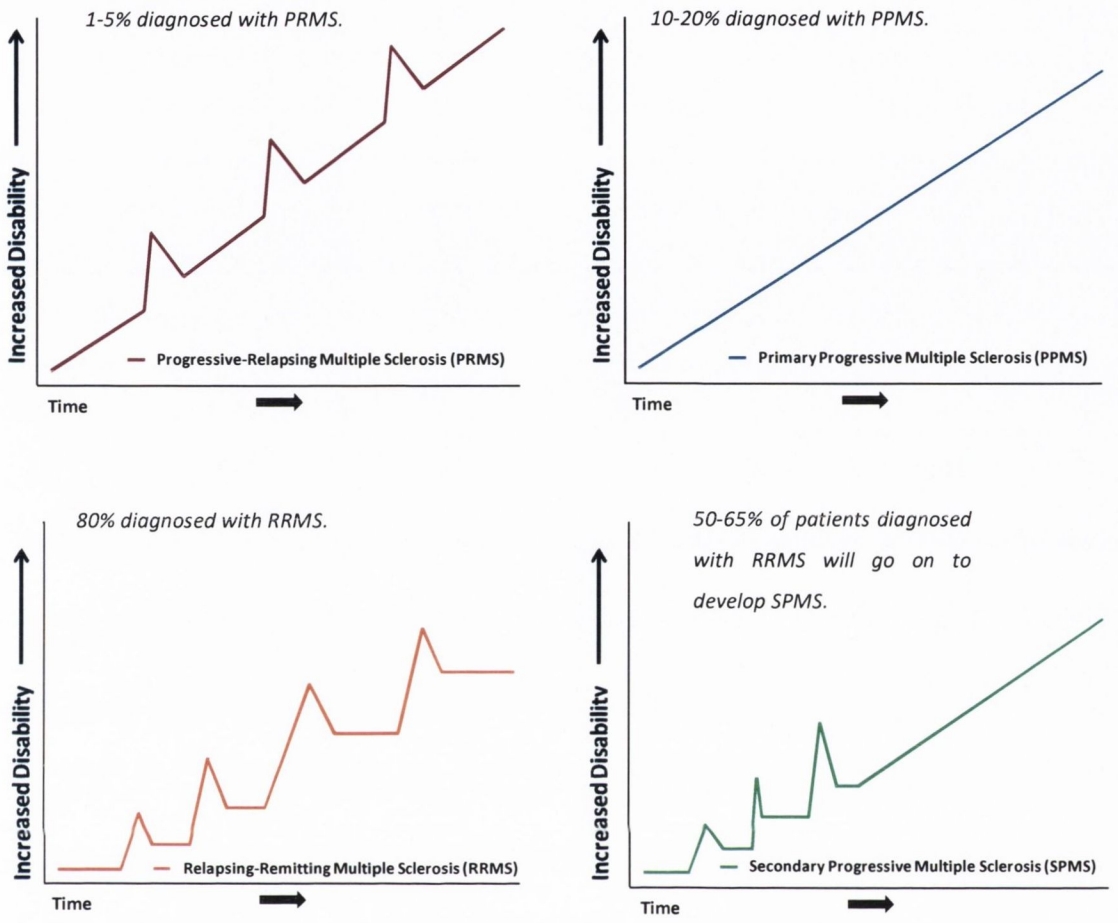


Figure 1.1 *The progression of Multiple Sclerosis.*

There are four internationally recognised forms of MS characterised by their frequency, intensity and progression. PRMS (primary relapsing multiple sclerosis) is characterised by a progression in the level of disability followed by latent periods where some recovery may take place. In contrast, PPMS (primary progressive multiple sclerosis) involves a steady increase in disability with no apparent "attacks" and no remissions. RRMS (relapsing remitting multiple sclerosis) is the most common of the subtypes, characterised by relapses followed by periods of remission, where there is full or partial symptomatic recovery (Pilz et al., 2008). SPMS (secondary progressive multiple sclerosis) occurs when a patient is initially diagnosed with RRMS, but experiences a gradual worsening of the disease with fewer remission periods, until the disease becomes entirely progressive.1.1.2 Diagnosis and prevalence of MS.

The diagnosis and progression of MS is unpredictable, making it difficult to treat and manage. There is no single test for MS. A combination of magnetic resonance imaging and a battery of neurological tests including nerve conduction studies usually leads to diagnosis of MS (Waubant, 2012). This is in accordance with the diagnostic criteria termed 'McDonald criteria'. A lumbar puncture can be used to eliminate other diseases and can indicate chronic inflammation, while the presence of oligoclonal bands in a patient's cerebrospinal fluid is an important disease indicator. The Expanded Disability Status Scale (EDSS) introduced by J.F Kurtzke in 1983 is a method of quantifying the degree of disability in MS according to clinical symptoms (Pilz et al., 2008). MS is usually diagnosed in patients between the ages of 20 and 50. The prevalence of MS, while difficult to establish is estimated to range between 2 and 150 per 100,000 depending on the specific country and population type. In Europe the prevalence is estimated to be around 83 per 100 000 with a female to male ratio of 2. There are an estimated 400,000 MS patients in Europe alone; a recent report showed a prevalence estimate of 168 per 100 000 in Northern Ireland (Pugliatti et al., 2006; Rotstein et al., 2006).

1.1.3 The progression and mechanism of Multiple Sclerosis.

Humans come in contact with bacterial peptides or viral particles on a regular basis. These can have sequences that closely resemble that of myelin antigens. In a phenomenon known as molecular mimicry, foreign antigens can cause cross-activation of autoreactive B- or T-cells (Chastain and Miller, 2012). Evidence exists indicating that myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocytes glycoprotein (MOG) are targets for an autoreactive immune response (Hellings et al., 2001). It is possible that a defective or leaky BBB plays a role in the onset of clinically recognisable MS by exposing the brain tissue, and in particular myelinated axons to innate circulating autoreactive T-cells (Palmer, 2010). This can then lead to a cascade of inflammatory events propagated by a largely $CD4^+$ and $CD8^+$ Th1 or Th17 immune response, contributing to multifocal tissue damage (Bruck, 2005). Local release of pro-inflammatory cytokines may also further promote demyelination. In addition, macrophages can become activated and contribute to the pool of damaging cytokines, while phagocytosing the myelin debris (Hendriks et al., 2005). Axonal degeneration ensues, the eventual outcome of which is the wide range of symptoms described for MS (Sospedra and Martin, 2005). This largely immunocentric view of MS however does not explain the observed continual axonal degeneration in the more progressive forms of the disease. Progressive forms of MS are not associated with inflammation or any notable involvement of the immune system and are primarily thought to be neurodegenerative in origin or due to a failure in

remyelinating processes (Hagemeier et al., 2012). These various forms of MS thus require different therapeutic treatments and clinical managements.

1.1.4 The genetics of Multiple Sclerosis.

As described previously the causes of MS are largely unknown. Currently it is thought exposure of genetically susceptible individuals to various environmental factors such as place of birth, distance from the equator, Epstein–Barr virus (EBV) infection late in life, childhood vitamin D deficiency or cigarette smoking may lead to disease development. While MS is not a classical hereditary disease there is a 2% risk of a child developing MS if their parent has the disease, likewise there is a 5% chance of a sibling developing the condition. In addition, a number of twin studies from several populations indicate that a monozygotic twin of an MS patient has a 30% risk of developing MS. In contrast dizygotic twins have only a 5% risk, this indicates the presence of a significant, but complex, genetic aspect in the conference of disease susceptibility (Willer et al., 2003). There are a number of candidate genes that can cause a predisposition to the development of MS. Most of these genes are involved in immune function, such as the gene for the IL-2α receptor found on chromosome 10p15 (Hafler et al., 2007). Interestingly single nucleotide polymorphisms (SNPs) in the IL-2α receptor gene confer susceptibility to not only MS, but also other autoimmune diseases such as type I diabetes, Graves disease and rheumatoid arthritis (Maier and Hafler, 2008). Another MS associated risk gene is the gene for the IL-7 receptor. The SNP (T2441) is found on exon 6 of the gene and leads to increased production of the soluble form of the IL-7 receptor. The physiological consequences of this remain unknown (Gregory et al., 2007). However, the strongest, consistent genetic risk for MS to date, with approximately a three-fold increased relative risk, can be seen in the human leukocyte antigen (HLA) chromosomal region. Association with this particular locus can be seen across nearly all populations studied and in patients with both RRMS and PPMS (McDonnell et al., 1999; Oksenberg et al., 2004). The HLA region encodes for major histocompatibility complex (MHC) proteins. These polymorphic cell membrane expressed glycoproteins, are among other things, heavily involved in coordinating the immune system's recognition of non-self from self antigens (Watts, 2004). Genome wide association (GWA) studies have greatly increased the understanding of the genetic aspect of MS providing numerous susceptibility genes. It will become increasing important however to provide functional evidence that connects genetic variability, with disease physiology, in the hope of developing new drug targets.

1.1.5 Current treatments for Multiple Sclerosis.

MS is a heterogeneous disease with symptoms being patient specific. Consequently the therapies and treatments devised to manage the disease are varied. MS treatment primarily aims at returning function after an immune attack, preventing new immune attacks, and preventing or reducing disability. During acute relapses the primary aim is to treat symptoms where most patients are hospitalised at this time. Glucocorticosteroids remain the gold standard for treatment of acute relapses (Hutchinson, 2009; Rhen and Cidlowski, 2005). Glucocorticosteroids inhibit transcription of pro-inflammatory cytokines, such as interleukin 1/2 (IL-1/2) and tumour necrosis factor-α (TNF- α), thus exerting an overall immunosuppressive effect. This particular therapy is commonly administered as intravenous methylprednisolone. It is well documented that glucocorticosteroids are highly effective in limiting the impact of a relapse in MS; however, they have no effect on the course or progress of the disease. While glucocorticosteroids remain important in the short term treatment of relapses, their side effects limit their use as a long-term treatment or management of the disease (Perumal et al., 2008). With the introduction of the Interferon (IFN) family of drugs and glatirarmer acetate (GA) in the early 1990's, the focus of MS treatment switched from 'immunosuppressive' to what is termed 'immunomodulatory'. IFN-β-1β (Betaferon®, Betaseron®), IFN-β-1α (Avonex®, Rebif®) and glatiramer acetate (GA) (Copaxone®) make up what is now considered 'basic therapy' for the treatment of MS (McCormack and Scott, 2004; Rovaris et al., 2007). The IFN-β's mode of action is complex and not completely understood. Their therapeutic effect is thought to be mediated by their effect on the immune system. IFN-β's have been shown to reduce expression of both major histocompatibility complex II (MHCII) and co-stimulatory molecules (e.g., CD80 and CD28) on antigen presenting cells (APCs) and lymphocytes. Collectively this reduces the likelihood of T-cell activation (Airas et al., 2007). IFN-β treatment also shifts the profile of the immune response away from Th1, towards a more Th2 mediated profile. Thereby reducing the production of pro-inflammatory cytokines (e.g., IL-6, IL-12, and IFN-γ) and increasing the production of anti-inflammatory cytokines (e.g., IL-4 and IL-10) (Noronha et al., 1993). Finally IFN-β treatment leads to a decrease in surface expression of VLA-4 (Very Late Antigen-4) on T-cells plus a shedding of sVCAM-1 (soluble vascular cell adhesion protein-1) molecules, resulting in a decoy mechanism and, thereby, a reduction in the cell surface VLA-4/VCAM-1 interaction. Collectively this can reduce the likelihood of T-cells crossing the BBB and causing damage to the brain parenchyma (Jensen et al., 2005). A common problem encountered with IFN-β treatment is the development of neutralizing antibodies, resulting in a loss of efficacy (Tourbah and Lyon-Caen, 2007).

Glatirarmer acetate (Copaxone®) is a preparation of synthetic polypeptides composed of four amino acids closely related to MBP (myelin basic protein) a suspected auto-antigen in MS. In reality GA's mechanism of action is not fully understood. It is not clear what cells or molecules are the main pharmacological target of GA. Again, GA appears to alter the profile of the immune response away from a Th1 to a more Th2 mediated profile. This can be beneficial as Th2 cells can stimulate antibody producing, repair enhancing B-cells, initiating a humoral immune response. This contrasts with the classic Th1 response, which is inflammatory by nature and eventually leads to an immune-mediated injury to the myelin sheath. GA also exerts an immunomodulatory effect on various APCs including monocytes (Neuhaus et al., 2001; Weber et al., 2007). GA is not affected by neutralizing antibodies; however injection site reactions are a problem encountered in GA users. Basic therapy has been shown to improve the clinical course of MS by reducing the relapse rate by ~30%, by alleviating relapse severity and by delaying the progression of disability in RRMS patients (Rovaris et al., 2007; Schwid and Panitch, 2007). It is important however to realise that these therapies do not affect the risk of relapses, and are treatments that do not alter the disease endpoints (Ruggieri et al., 2007). The relatively newer MS therapy natalizumab (Tysabri™) is licensed as a monotherapy for highly active RRMS. Natalizumab is a monoclonal antibody (mAb) directed against the adhesion molecule α-4 integrin. The α4β1 integrin VLA-4 is expressed on the cell surface of leukocytes and its interaction with the adhesion molecule VCAM-1 found on endothelial cells is essential for the entry of leukocytes to the brain parenchyma. The antibody sterically inhibits the VLA-4/VCAM-1 interaction thereby blocking and attenuating lymphocyte interaction, tethering and trafficking of the cell across the BBB. Monthly infusions of the drug resulted in a significant decrease in the risk of progression of disability for patients, coupled with improved MRI measurements (Rommer et al., 2008). Side-effects associated with natalizumab include infusion reactions and some hypersensitivity reactions. Patients have also presented with fatigue upon treatment with natalizumab, but in the main the drug is well tolerated. Natalizumab is however associated with an increased risk of developing a rare CNS disease: progressive multifocal leukoencephalopathy (PML) (Bloomgren et al., 2012). PML is an opportunistic infection caused by the JC virus, generally associated with patients that are immunocomprised. A pathogenic form of the JC virus causes widespread destruction of oligodendrocytes and subsequent development of numerous demyelinating plaques (Weissert, 2011). Following the development of 3 cases of PML during clinical trials (incidence of approx. 1 in 1000) natalizumab was temporarily withdrawn from the market, only to be re-released in 2006 (Yousry et al., 2006). The beneficial effects and the improved quality of life experienced by patients on natalizumab make this an important therapy (Brown, 2009; Linker et al., 2008). Other MS

therapies include mitoxantrone (Novantrone®), which is an approved treatment for worsening RRMS or SPMS. Mitoxantrone is a type II topoisomerase inhibitor, thereby inhibiting DNA repair and synthesis. Despite it being potentially more toxic than the IFN-β's, it is an approved second-line treatment for aggressive RRMS and SPMS (Fox, 2006; Rieckmann et al., 2004). Cyclophosphamide (Cytoxan™) is an alkylating agent which binds to DNA and disrupts mitosis, resulting in an overall immunosuppressive effect. Cyclophosphamide usage is limited due the wide range of side effects associated with it (Weiner and Cohen, 2002). Intravenous immunoglobulins (IVIGs) and the purine synthesis inhibitor azathioprine also come under the heading of 'basic therapy', although efficacy issues and possible teratogenic/carcinogenic effect respectively limit their usage. Patients exhibiting reduced response to basic therapy or high relapse frequency are usually switched to 'escalating therapy' (Rieckmann et al., 2004). Collectively these drugs encompass basic and escalating therapies for the treatment of MS. Whilst a lot of progress has been made in recent years, MS remains without a cure; the above drugs are only capable of managing the disease and its symptoms. To date all MS therapies are administered by daily, weekly or monthly injections which can cause discomfort, complications with injection site reactions and a general reduction in quality of life. Consequently developing new oral therapies occupy most pharmaceutical companie's MS related activities.

1.1.6 Future treatments of Multiple Sclerosis.

There is urgent need in MS therapies for compounds with superior efficacy matched with better long-term safety profiles, increased ease of administration and improved tolerability. In addition there is no approved therapy for both progressive forms of the disease. There are currently a large number of emerging therapies for MS which will attempt to address these unmet needs. Encouraging phase III trial results have led to a number of companies applying for FDA (Food and Drug Administration) and EMA (European Medicines Agency) approval for new MS therapies, with further applications forecast for 2012/2013 (**Fig.** 1.2). These compounds include humanized monoclonal antibodies (mAbs) which target lymphocyte antigens leading to immunosuppression. For example alemtuzumab (Lemtrada®) is being developed by Genzyme and is an anti-CD52 mAb, currently approved for the treatment of chronic lymphoid leukaemia. Alemtuzumab is a once yearly infusion which results in a rapid reduction in numbers of CD52 positive B- and T-lymphocytes, monocytes, macrophages, NK cells and some granulocytes (Coles et al., 2012). Daclizumab (Zenapax®) (marketed in Europe by Hoffmann-La Roche for the prevention of rejection in organ transplantation) is undergoing phase III trials as a possible MS therapy. Daclizumab is a mAb that targets CD25, the alpha subunit of the IL-2 receptor, treatment leads to a reduction in $CD8^+$ cells

with an observed expansion of regulatory $CD56^+$ NK cells (Bielekova et al., 2009). Hoffmann-La Roche also has the mAb ocrelizumab in phase III clinical trials for the treatment of MS. Ocrelizumab is an anti-CD20 mAb, treatment with which leads to the antibody and complement dependent lysis of $CD20^+$ B-cells (Chaudhuri, 2012). Laquinimod, teriflunomide and dimethyl fumarate are new oral compounds that show positive results in the reduction of Gd-enchancing lesions and brain lesion activity (Pilz et al., 2008; Yang et al., 2004). While laquinimod, teriflunomide and dimethyl fumarate appear to be well tolerated, they have obscure mechanisms of action. Laquinimod is a derivative of Teva Pharmaceutical's failed experimental immunomodulator linomide. The FDA has granted this compound a fast track status due to its promising effects on lesion activity, disability progression as well as an observed reduction in relapses. Laquinimod appears to work by causing a shift the Th1 immune response to a more Th2 mediated response (see **Chapter** 5) (Rammohan, 2010). Dimethyl fumarate (BG-12) is a fumaric acid ester that appears to up regulate the nuclear factor-E2-related factor 2 transcriptional pathway. Nuclear factor-E2-related factor 2 is a master regulator of the antioxidant response which is important in protection against oxidative stress (Itoh et al., 1997). Another possible mechanism of action of BG-12 is the inhibition of the pro-inflammatory $NF\text{-}_\kappa B$ pathway. BG-12 showed promising efficacy coupled with a favourable safety profile in phase II trials, currently BG-12 is undergoing phase III trials (Kappos et al., 2008). Teriflunomide (Aubagio®) and cladribine (Mylinax®) are also in phase III trials for the treatment of MS. Teriflunomide inhibits the *de novo* synthesis of pyramidines resulting in a reduction in B- and T-cell proliferation (Korn et al., 2004). Cladribine is a purine analog which is immunosuppressive in its action, leading a reduced lymphocyte count (Brousil et al., 2006). We are particularly interested in the newly FDA approved compound FTY720-P (Fingolimod™) (Gilenya®). FTY720-P is an orally available novel immunomodulator which is a metabolite of myriocin, derived from the fungus *isaria sinclairii* (Hughes, 2009). FTY720-P exerts its pharmacological effects through a set of G-protein coupled receptors (GPCRs) termed sphingosine-1-phosphate receptors (S1P receptors) (Chiba, 2005). These receptors exhibit a diverse tissue and cellular expression profile and activate specific signalling pathways through their coupling to various G-proteins. A subset of these receptors, the sphingosine-1-phosphate 1 receptors (S1P1 receptors), are found on the glial cells astrocytes in the CNS. The focus of this study is to determine the role of S1P1 receptors in astrocytes in the brain.

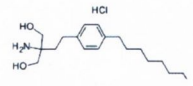
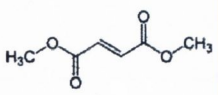
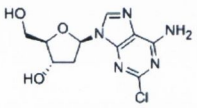
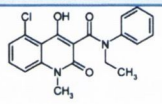
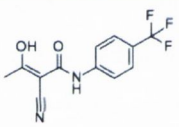
Drug	Company	Description	Structure
Fingolimod Gilenya® (granted approval)	*Novartis*	An S1P1 receptor agonist that alters lymphocyte trafficking thereby exerting an anti-inflammatory effect.	
Dimethyl fumarate BG-12® (Phase III)	*Biogen Idec*	Active metabolite methyl - hydrogenfumarate activates the transcription factor Nrf2 and endogenous antioxidant factors.	
Cladribine Movectro® (granted approval)	*Merck Serono*	A purine analog that inhibits DNS synthesis, is immunosuppressive in its action, leading a reduced lymphocyte count.	
Laquinimod N/A (Phase III)	*Teva/Active Biotech*	Is a quinoline derived immune-modulator with an obscure mode of action.	
Teriflunomide Aubagio® (in review)	*Sanofi-Aventis*	The active metabolite of leflunomide, an inhibitor of the synthesis of pyramidines resulting in a reduction in B- and T-cell proliferation.	
Ocrelizumab N/A (Phase III)	*Genentech/ Biogen*	A recombinant, mab which targets $CD20^+$ B-cells, leading to antibody and complement dependent cell lysis.	*Antibody*
Daclizumab Zenapax® (Phase III)	*Hoffmann-La Roche*	An anti-CD25 mab that reduces aberrant T-cell activation and increases regulatory NK cell numbers.	*Antibody*
Alemtuzumab Lemtrada® (Phase III)	*Sanofi-Aventis/ Genzyme*	An anti-CD52 mab. Treatment leads to depletion of $CD52^+$ T-cells, B-cells, NK cells, macrophages and monocytes.	*Antibody*

Figure 1.2 *A review of new disease modifying therapies for the treatment of MS, recently released or undergoing late stage phase III clinical trials.*

1.2 Sphingosine-1-phosphate receptors.

1.2.1 Sphingosine-1-phosphate: A bioactive sphingolipid.

Sphingosine-1-phosphate (S1P) is a bioactive, blood borne sphingolipid metabolite implicated in a diverse range of biological processes. S1P is produced intracellularly as part of natural sphingolipid metabolism, which begins with the formation of 3-keto-dihydrosphingosine from palmitoyl-CoA and serine by the ER anchored enzyme serine palmitoyltransferase, and ends with the phosphorylation of sphingosine to sphingosine-1-phosphate in the cytosol (**Fig.** 1.3). Receptors for S1P were first discovered in 1998 (Lee et al., 1998). The receptors for S1P, (of which there are five subtypes, S1P1-5) are expressed ubiquitously throughout the body (Chun et al., 2002). These GPCRs are coupled to various G-proteins and regulate numerous downstream signals, which explains the wide variety of biological responses attributed to S1P signalling (Brinkmann, 2007). Cellular S1P levels are tightly regulated by the balance between its synthesis by sphingosine kinase phosphorylation of sphingosine and its irreversible degradation by S1P lyase, or its reversible dephosphorylation back to sphingosine, by S1P-phosphatases (**Fig.** 1.4). The concept of a sphingolipid 'rheostat' was developed to explain the apparent opposing affects of the S1P precursors ceramide and sphingosine with S1P on cell fate (Hait et al., 2006). S1P is anti-apoptotic, promotes growth and cellular survival in contrast to ceramide and sphingosine which are increased in response to stress stimuli and are pro-apoptotic and anti-mitogenic (Cuvillier et al., 1996). The levels of the two isoforms of sphingosine kinase, SphK1 and SphK2 are tightly regulated in the cell and can be stimulated by a wide range of extracellular signals (Siow et al., 2011). Most noticeable of these are the growth factors and cytokines, including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Spiegel and Milstien, 2003). SphK1 is the more widely studied and has been shown to be the major determinant of S1P levels. Activation of SphK1 requires phosphorylation by ERK2 which leads to translocation and association of SphK1 with the plasma membrane. This allows the enzyme to come in close contact with its substrate, sphingosine. Activation of SphK1 leads to localised S1P production and secretion, allowing S1P to act as a ligand in an autocrine fashion, or on nearby cells in a paracrine fashion through its five receptors. This has been observed during PDGF induced cellular migration and has major implication for vascular maturation (Hobson et al., 2001). Platelets actively secrete S1P at areas of inflammation and tissue damage to aid angiogenesis and to establish an S1P gradient (English et al., 2000). Many cells are capable of producing and secreting S1P, however the main producers of S1P are erythrocytes and endothelial cells (Pappu et al., 2007).

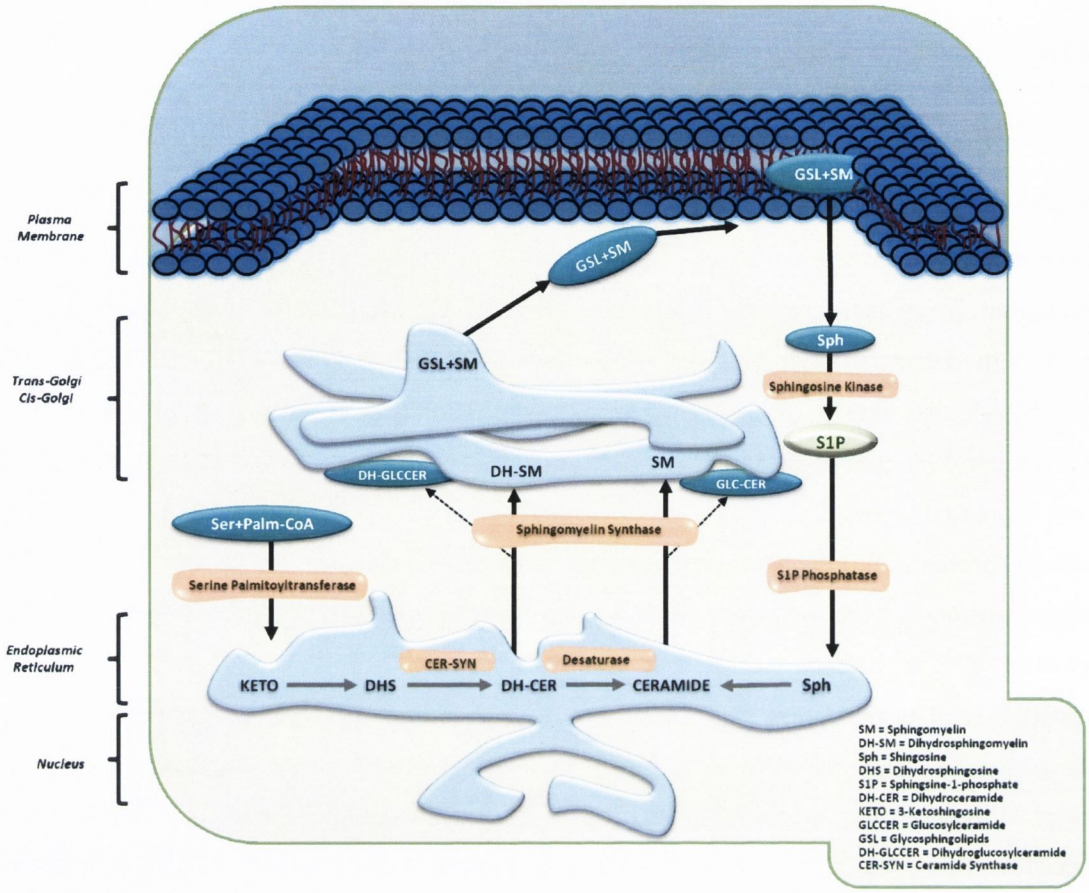


Figure 1.3 *An overview of sphingolipid metabolism.*

The *de novo* production of all sphingolipids starts in the ER with the condensation of the amino acid L-serine and palmitoyl CoA. Following a complex set of reactions the newly formed dihydroceramide and/or ceramide are translocated to the Golgi apparatus and converted to sphingomyelin or dihydrosphingomyelin. The newly converted complex glycosphingolipids are inserted into the plasma membrane. Sphingosine is produced via the degradation of plasma membrane GSLs, the signalling sphingolipid S1P is produced in the cytosol as a result of the phosphorylation of sphingosine by sphingosine kinases.

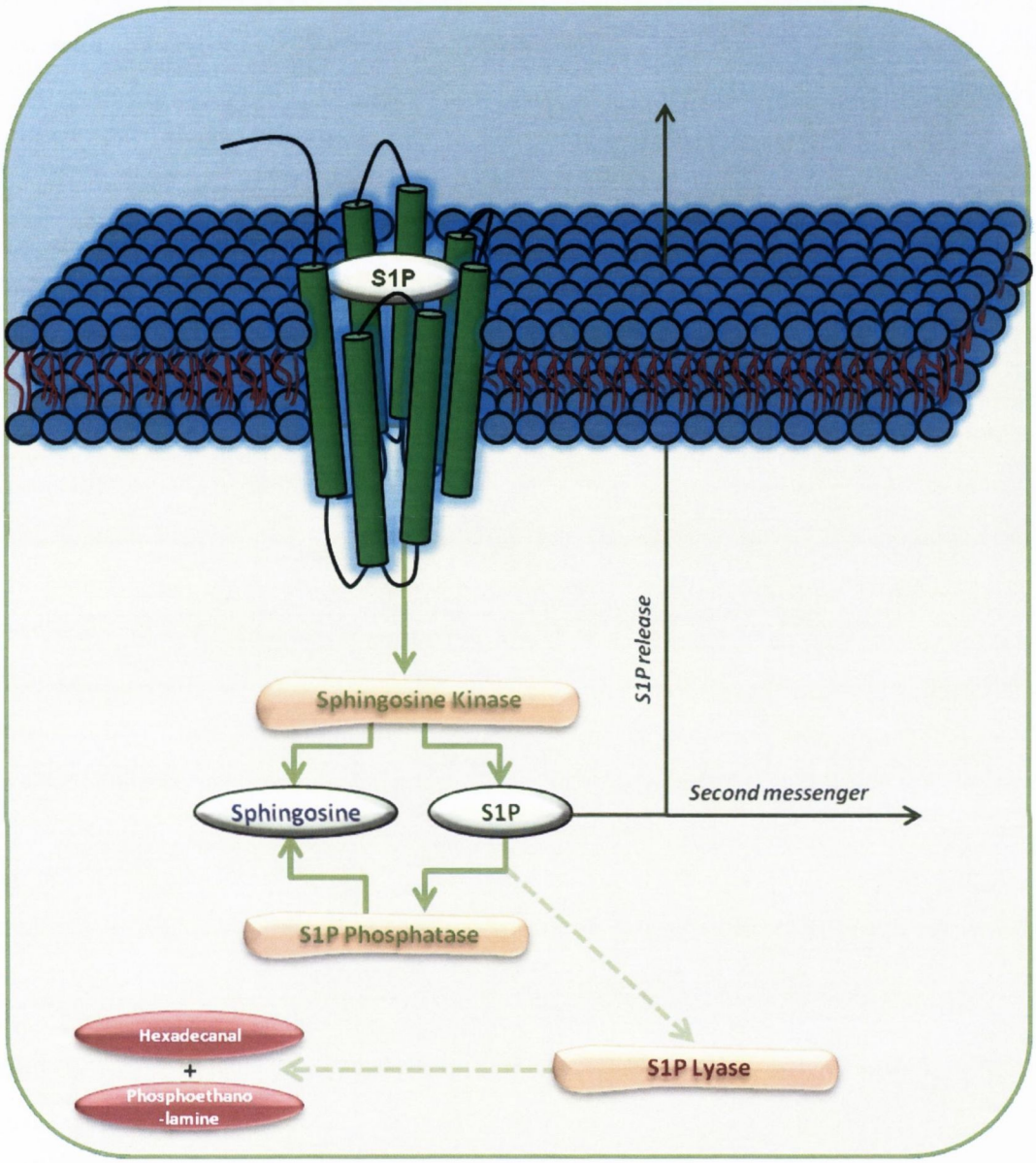


Figure 1.4 *An overview of sphingosine-1-phosphate production and degradation.*

S1P is produced intracellularly by the phosphorylation of sphingosine by sphingosine kinases. S1P is secreted and acts in an autocrine fashion through one of five GPCRs or in a paracrine fashion on nearby cells. S1P is also a putative second messenger, with definitive intracellular targets yet to be discovered. S1P is converted back to sphingosine through the enzyme S1P phosphatase or irreversibly broken down into hexadecanal and phosphoethanolamine by S1P lyase.

1.2.2 S1P as a biomarker for disease.

Observed changes in S1P levels in both plasma and CSF which appear to correlate to disease activity have marked S1P out as a possible biomarker for a number of neurological and non-neurological diseases. Levels of S1P and a number of sphingolipid metabolites have been measured in the brains of Alzheimer's disease (AD) patients (He et al., 2008). Briefly, significant elevations in both ceramide and sphingosine were observed in AD patients compared to age matched controls, this correlated with a significant reduction in S1P levels. The elevated levels of acid sphingomyelinase (ASM; the enzyme responsible for conversion of sphingomyelin to ceramide) and reduced S1P levels in AD were also seen to correlate with levels of amyloid beta (Aβ) and hyperphosphorylated tau protein, both pathological markers of AD (He et al., 2008). Thus it is possible that S1P and its sphingolipid precursors may not only be contributing to the pathologic processes of AD, but also may be utilised as marker for Alzheimer's disease activity. Plasma levels of S1P have been shown to be increased in animal models of type I diabetes. In addition to this it has been shown that treatment with an S1P2 receptor antagonist (JTE-013) can prevent the onset of streptozotocin-induced diabetes via a mechanism involving the protection of islet β-cells (Imasawa et al., 2010). Furthermore S1P2 receptor knockout animals showed reduced β-cell apoptosis in combination with lower blood glucose levels (Imasawa et al., 2010). Studies have shown that SphK1 mediated production of S1P confers tumour resistance to apoptosis, promotes tumour growth, angiogenesis and metastasis (Pyne and Pyne, 2010). A recent study of patients with late stage prostate cancer revealed that levels of circulating S1P were significantly reduced in these patients compared to age matched healthy controls and patients suffering with benign prostate hyperplasia (Nunes et al., 2012). The data suggests that a decrease in circulating S1P, or down regulation of erythrocyte SphK1 activity during progression of prostate cancer are potential new biomarkers for this disease. Interestingly, a study has recently described a new positive feedback loop involving the transcription factor, signal transducer and activator of transcription 3 (STAT3), IL-6 and the S1P1 receptor (Lee et al., 2010). Data shows that S1P1 receptor activation promotes persistent activation of STAT3 in tumour cells. Increased S1P1 receptor expression activates STAT3 and up-regulates expression of the pro-inflammatory cytokine IL-6 which is important for STAT3 activation and tumour development (Lee et al., 2010). In light of S1P and SphK1 involvement in cancer, a number of SphK inhibitors are currently being developed. One such inhibitor is SK1-I (BML-258), which has been shown to inhibit the growth of both leukaemia and glioblastoma tumour cells in xenografts (Kapitonov et al., 2009; Paugh et al., 2008).

1.2.3 S1P mediated signalling.

As stated above, S1P signalling is implicated in a wide range of biological responses as a result of its actions on five distinct GPCRs; it also thought that S1P may act as an intracellular messenger. Excretion pathways of S1P to the extracellular environment are poorly understood but appear to involve the ATP-binding cassette transporter (ABCC1) to translocate S1P across the membrane, as is the case in mast cells (Mitra et al., 2006). Serum levels of S1P are in the mid-nanomolar range, while levels in the interstitial fluid are ~1000 fold less. Most blood borne S1P is bound to chaperones such as albumin, low- and in particular high-density lipoproteins (HDL) (Murata et al., 2000b). Research has shown that HDL-bound S1P is active and can associate and dissociate from HDL to interact with its receptors (Matsuo et al., 2007). S1P was first presumed to be an intracellular signalling molecule; however, definitive intracellular targets for S1P have yet to be identified. Work done on yeast which do not express GPCRs showed that long chain sphingoid bases regulate response to heat stress. In addition, plants which do not express S1P receptors showed that S1P regulated stomatal aperture (Coursol et al., 2005; Jenkins and Hannun, 2001). However the most convincing evidence for S1P as an intracellular second messenger comes from work done examining mobilisation of Ca^{2+}. Intracellular application of S1P has also been shown to cause store operated Ca^{2+} entry (SOCE) in vascular smooth muscle cells, in a receptor independent manner (Hopson et al., 2011). However the molecular mechanisms of this novel signaling pathway remain unknown. In addition to mobilisation of Ca^{2+}, inhibitors of sphingosine kinases, the enzymes responsible for the generation of S1P from sphingosine inhibit growth factor induced cellular proliferation and survival (Olivera and Spiegel, 2001). PDGF has been shown to cause movement of sphingosine kinases to the nuclear envelope, implying S1P may play a role in the nucleus, although there is as yet no direct evidence to support this theory (Olivera et al., 1999). Molecular targets for intracellular S1P have yet to be identified, thus it remains to be seen if S1P can act as a classical second messenger or in a more metabolic role. S1P was initially found to be the natural ligand for the orphan GPCR, endothelial differentiation gene 1 (EDG1) now known as the S1P1 receptor (Lee et al., 1998). The large EDG family of receptors can be split into two separate groups, the S1P receptors and the lysophosphatidic acid (LPA) receptors which have the phospholipid derivative, lysophosphatidic acid (LPA) as their natural ligand (Chun et al., 2002). The EDG/S1P receptor subgroup comprises five members (S1P1-5). Signalling of the S1P receptor subfamily plays a role in cellular migration, survival, differentiation and proliferation (**Fig.** 1.5) (Hla and Brinkmann, 2012).

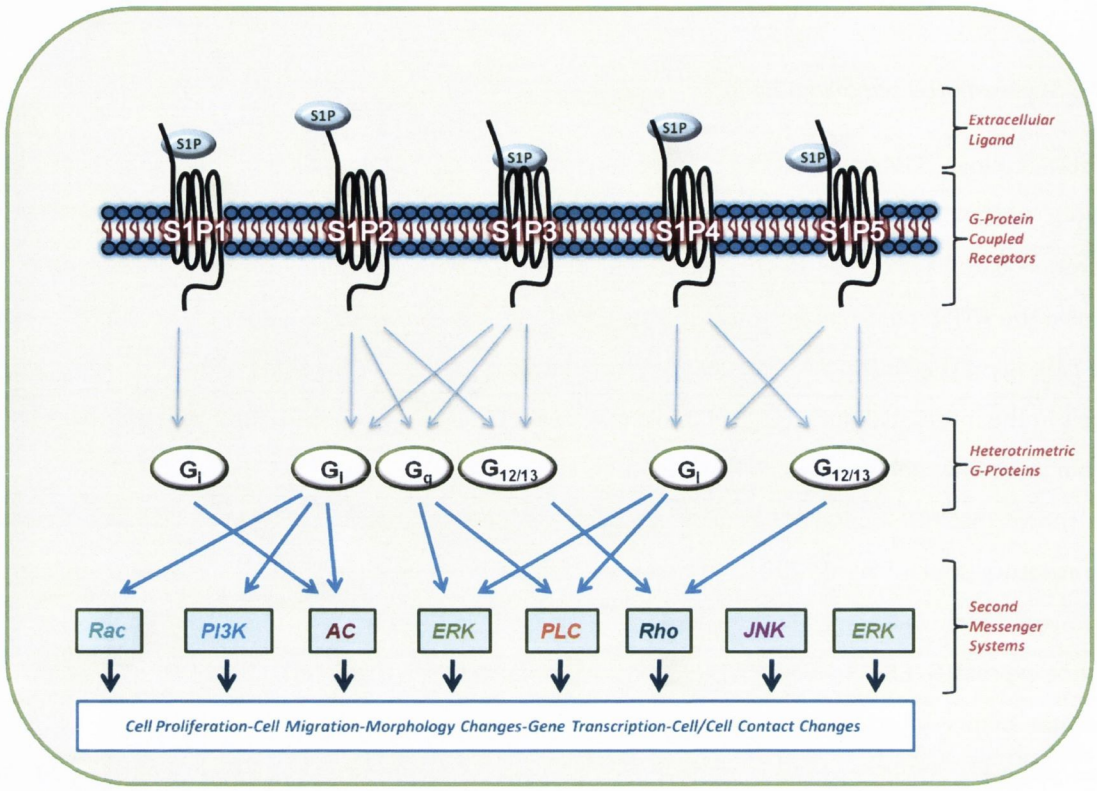


Figure 1.5 *A schematic depicting the complexity of S1P signalling.*

Extracellular S1P exerts its effect on cells via five known GPCRs (S1P1-5) found to varying levels is all tissues of the body. These S1P receptors are coupled to various heterotrimetric G-proteins, all five receptors are coupled to G_i and all except S1P2 can couple to G_{12}. These G-proteins in turn signal to many downstream effector proteins. These include phospholipase C and D (PLC and PLD), extracellular-signal-regulated kinases (ERKs), adenylate cyclase (AC), and c-Jun N-terminal kinases (JNK).

1.3 Fingolimod (FTY720-P) (Gilenya®)

1.3.1 Development of new immunomodulatory compound FTY720.

Gilenya® (Fingolimod; FTY720) [2-amino-2(2-(4-octylphenyl) ethyl)-propan-1, 3-diol] is an orally available, novel immunosuppressant drug produced by Novartis AG (Basel, Switzerland). It has recently completed two phase III clinical trials and has been granted FDA approval as a first-line therapy for RRMS (Kappos et al., 2010). FTY720 was first synthesized in 1992 as a metabolite of myriocin, an antibiotic derived from a type of thermophilic fungi known as *isaria sinclairii* (Adachi and Chiba, 2008). FTY720 is a pro-drug that is phosphorylated to FTY720-P *in vivo*. FTY720 is phosphorylated by two conserved enzymes, sphingosine kinase 1 and 2 (SphK1 and SphK2) both of which play a major role in sphingolipid metabolism (**Fig.** 1.4). Sphk2 has been found to be the enzyme predominately responsible for the phosphorylation of FTY720 *in vivo* (Billich et al., 2003). FTY720-P is a structural analogue of S1P (**Fig.** 1.6) and exerts its pharmacological actions through the same receptors as S1P. The drug is a potent nanomolar agonist of S1P1, S1P4 and S1P5, a partial agonist of S1P3 and has no effect on S1P2 receptors (Albert et al., 2005; Brinkmann et al., 2002). Gilenya® successfully came through two of the largest phase III clinical trials in MS (Kappos et al., 2010). The first 'FREEDOMS' trial was a double-blind, placebo controlled clinical trial, designed to evaluate the effects of varying daily doses of oral FTY720-P for the treatment of MS (Kappos et al., 2010). The second 'TRANSFORMS' trial was a 12 month, double-dummy trial used to compare oral doses of FTY720-P with interferon-β-1a in the treatment of RRMS (Cohen et al., 2010). Results from the clinical trials showed that both doses (0.5mg and 1.25mg) reduced the risk of disability progression and the relapse rate (54% and 60% respectively), compared to placebo. FTY720-P administered once daily also resulted in an improvement in MRI measures of MS (Horga et al., 2010). No significant difference in efficacy was observed between the doses, with the lower dose being tolerated better. Side effects encountered during the study included cardiovascular effects such as asymptomatic heart rate reduction and slight blood pressure increase. Other side effects included fatigue, macular oedema, and reversible elevation of liver enzymes. An increase in lower respiratory tract infections was documented in FTY720-P treated patients. Interestingly an increase in the risk of reactivation of latent herpes virus (with two fatal herpes infections) occurred with patients receiving the higher 1.25-mg dose of Gilenya®. While follow up studies are needed to fully establish the safety profile of the drug, regulatory approval for the 0.5mg dose of FTY720-P was applied for by Novartis through the US FDA and the EMA, and was granted in September 2010 (Kappos et al., 2010).

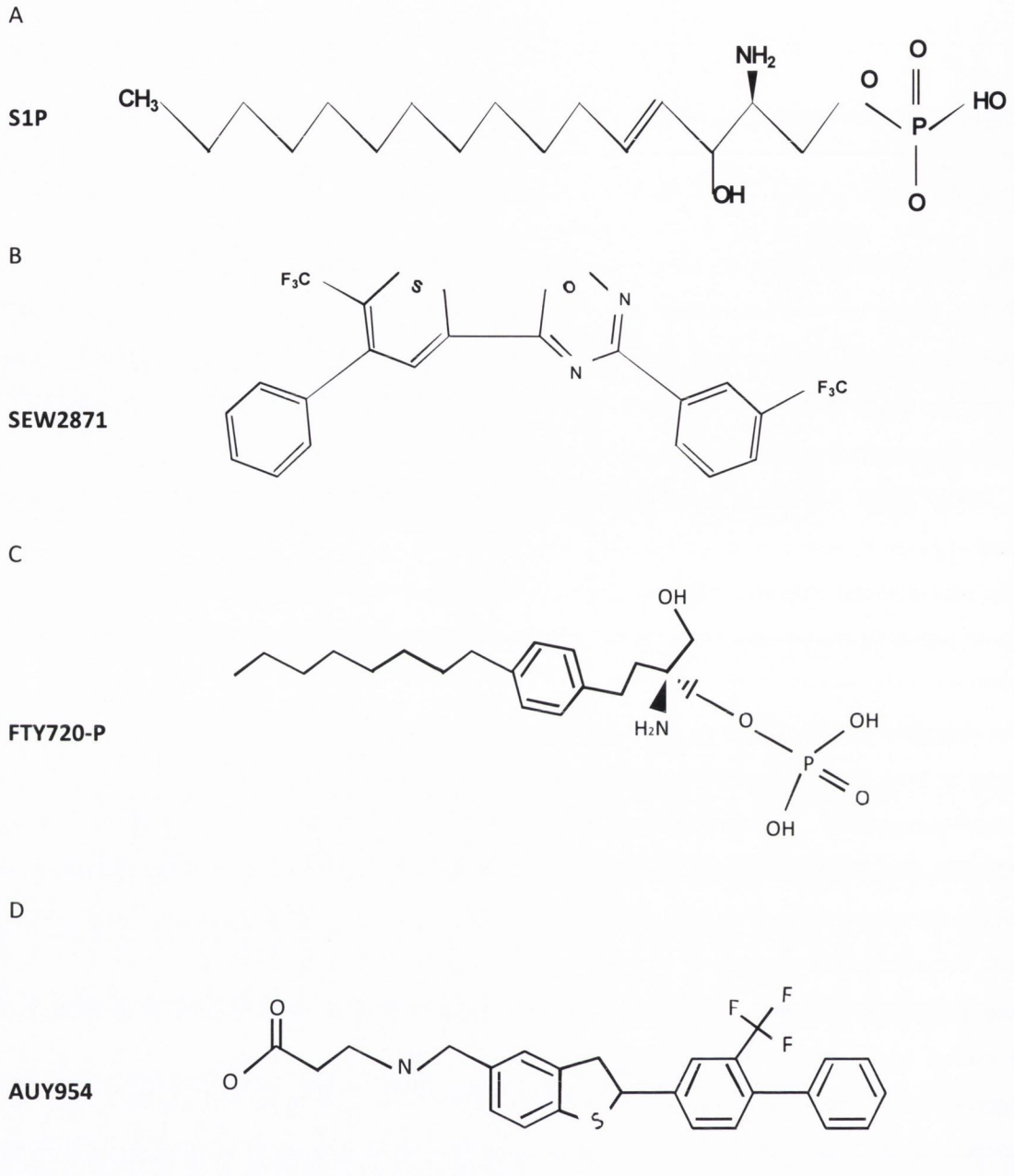


Figure 1.6 *Chemical structures of S1P receptor agonists.*

Chemical structures of **(A)** sphingosine-1-phosphate (S1P) a bioactive lysophospholipid, **(B)** SEW2871, an S1P1 receptor specific agonist, **(C)** FTY720-P, an immune modulator and analogue of S1P and **(D)** AUY954, an S1P1 receptor specific agonist.

1.3.2 Mechanism of action of FTY720-P.

The proposed mechanism of action for FTY720-P in the treatment of MS involves its effect on lymphocytes in secondary lymphoid organs (Chun and Hartung, 2010). FTY720-P binds to and causes rapid and sustained internalisation of membrane expressed S1P1 receptors (Mullershausen et al., 2009) (**Fig.** 1.7). FTY720-P induced inhibition of lymphocyte egress can be explained by selective action on S1P1 receptors. Deletion experiments in lymphocytes resulted in a reduced peripheral lymphocyte count similar to that observed upon FTY720-P treatment (Matloubian et al., 2004). When this internalisation occurs on lymphocytes it renders the cells incapable of sensing and responding to the vascular S1P gradient, a signal necessary for cellular emigration from the secondary lymphoid tissue (Allende et al., 2004). The attenuation of T-cell trafficking and resultant lymphopenia reduces the recirculation of autoreactive T-cells. This sequestration of lymphocytes in the lymphoid tissue reduces the likelihood of autoreactive T-cells entering the brain parenchyma and therefore reducing the likelihood of harmful inflammatory events (Brinkmann, 2009). Binding of both FTY720-P and S1P to the S1P1 receptor causes internalisation via the endosomal pathway. However, unlike S1P which causes recycling of the receptor, FTY720-P causes a persistent state of internalisation of the S1P1 receptor, followed by ubiquitination of the receptor and down-regulation at an mRNA level (Oo et al., 2007). This mechanism of action has now been termed 'functional antagonism' and this event impedes the pro-migratory/pro-inflammatory response of the S1P-S1P1 receptor axis. FTY720-P is advantageous as a drug for MS because it prevents lymphocytes from reaching sites of inflammation without creating a generalised state of immunosuppression. Other non-immune system related effects of FTY720-P have been observed and it is theorized that these might also contribute to efficacy of FTY720-P in the treatment of MS. FTY720-P agonism of S1P1 receptors inhibits both angiogenesis and vascular permeability (Sanchez et al., 2003; Schmid et al., 2007). This differs to the pro-migratory, pro-angiogenic effect of S1P. It was thought initially that tightening of the endothelial barrier in the lymphoid tissue essentially blocked the egress of lymphocytes from the lymph nodes. However, while this might contribute to the reduced entry of T-cells into the CNS, it is not the main cause of the lymphopenia (Mandala et al., 2002).

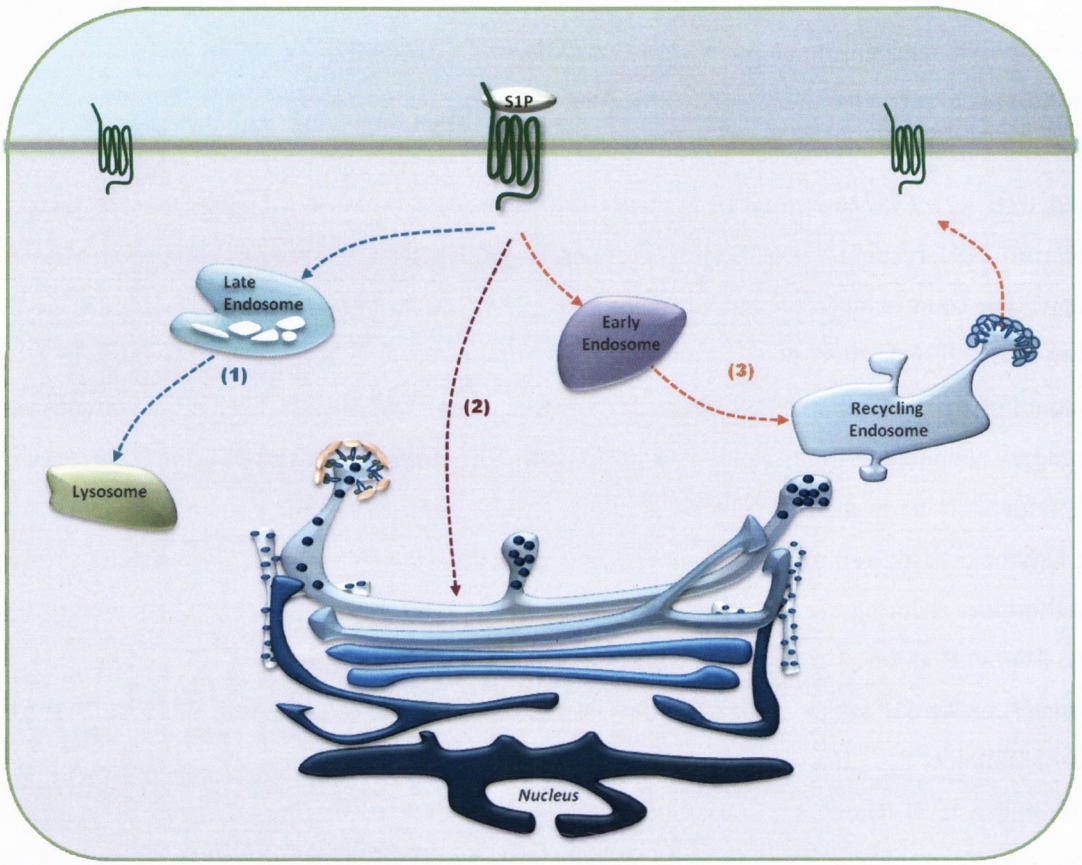


Figure 1.7 *Internalisation of G-protein coupled receptors.*

Ligand bound GPCRs generally follow two main pathways of internalisation. (1) Internalised vesicles fuse to form early endosomes. These endosomes mature to become late endosomes and fuse with lysosomes where the contents of the endosome are degraded. (2) The internalisation pathway of the S1P1 receptor when bound to FTY720-P. Receptors are almost exclusively found in the trans-Golgi-network (TGN) where they continue to signal (Mullershausen et al., 2009) (3) After early endosome production the vesicles fuse to recycling endosomes, where they are stripped of their ligand and recycled back to the plasma membrane (Marchese et al., 2003).

1.3.2 FTY720-P's widespread effect on the cells of the brain.

As previously discussed, the proposed mechanism of action for FTY720-P is an immunomodulatory mechanism based around the sequestering of pathologic lymphocytes in secondary lymphoid tissue. However, S1P receptors have a differential distribution throughout the CNS, with at least one of the five receptors being expressed in every cell of the CNS (Dev et al., 2008). FTY720 is highly lipophilic, due to its charged phosphate group. FTY720-P can cross the BBB easily and becomes localised to the CNS white matter (Foster et al., 2007). The CNS, and in particular the neural cells contain endogenous SphK2 activity capable of producing the active metabolite from the pro-drug, therefore it is probable that the phosphorylated version can be produced *in situ* (Billich et al., 2003). It has been shown that clinically relevant doses of orally administered FTY720-P are distributed to the CNS where levels of FTY720 and FTY720-P reach an equilibrate state (Billich et al., 2003). Preliminary studies show that i.c.v injection of FTY720-P in an EAE model leads to reduced disease severity without having an effect on peripheral lymphocyte counts (unpublished observations).

1.3.4 S1P receptors in Astrocytes.

Astrocytes express mainly S1P1 and S1P3 receptors, with some detectable levels of S1P2 mRNA (Bassi et al., 2006; Chae et al., 2004; Rao et al., 2004). FTY720-P has been shown to have numerous effects on astrocytes mainly through its interaction with the S1P1 receptor. Functional antagonism of S1P1 in astrocytes *in vivo* may alter their functional properties without affecting astrocyte number (Dev et al., 2008). It has been shown that there is a marked increase in local S1P levels following spinal cord injury. In addition, injection of S1P in mice CNS produces astrocytic proliferation synonymous with astrogliosis (Kimura et al., 2007; Sorensen et al., 2003). Astrocytes can produce S1P in response to a multitude of signals, most notable from growth factors such as bFGF and pro-inflammatory cytokines such as IL-6. S1P can act in an autocrine fashion through the S1P receptors, or as described earlier evidence also exists suggesting that S1P may play a role as an intracellular second messenger (Payne et al., 2002). Functional changes in astrocytes induced by FTY720-P may counteract the negative effect of S1P on astrocytic gap junctional communication. This may strengthen gap junctional communication of astrocytes with neuronal cells and with the endothelial cells of the BBB, which is disturbed during inflammatory events observed in MS (Giaume et al., 2010; Rouach et al., 2006). It is clear that both FTY720-P and S1P can modulate S1P receptors on astrocytes, the exact physiological effect of this has yet to be determined and is a primary research aim of this thesis.

1.3.5 S1P receptors in Microglia.

Microglia are the resident macrophages of the CNS and therefore contribute and participate in the inflammatory processes associated with MS (Raivich, 2005). Activated microglia are capable of secreting numerous neurotoxic substances, including reactive oxygen species, NO, pro-inflammatory cytokines and complement proteins (Rozemuller and van Muiswinkel, 2000). In the absence of T-cells, activation of resident microglia has been shown to be sufficient to instigate inflammatory demyelination. Microglial driven axonal damage has also been shown to represent an early stage of axonal injury prior to the formation of demyelinating plaques (Furtado et al., 2006; Marik et al., 2007). Loss of oligodendrocytes in the tissue surrounding expanding MS lesions seems to occur in the presence of activated microglia. It is this loss of oligodendrocytes that leads to the myelin loss (Henderson et al., 2009). Activated microglia play an important role in the pathogenesis of MS and are major constituents of demyelinating plaques (Sawada, 2009). Microglia express mostly S1P3 and S1P1 receptor mRNA (Dev et al., 2008). Similar to astrocytes S1P receptor expression is dependent on the activation state of the cell (Tham et al., 2003). The activation state of microglia is governed by their local environment and signals acting on the cells, for instance elevated S1P levels following inflammation or CNS trauma (Tham et al., 2003). It has been shown that during spinal cord injury activated microglia accumulate at the injury site, produce and release S1P, thereby contributing to an elevated local concentration of S1P (Kimura et al., 2007). The interaction between FTY720-P and microglia has yet to be fully characterized although it has been shown that FTY720-P is capable of inhibiting macrophage migration into inflammatory lesions (Miron et al., 2008b).

1.3.5 S1P receptors in Neurons.

Neurons mainly express S1P1, S1P2 and S1P3 receptors (Dev et al., 2008). It is believed that S1P signalling through S1P1 receptors and S1P2 receptors on neurons have opposing effects on neurite outgrowth (Toman et al., 2004). Activation of S1P receptors by S1P on dorsal root ganglion neurons differentially affects process extension induced by nerve growth factor (NGF) (Toman et al., 2004). S1P receptor signalling has also been implicated in pain (Xie et al., 2012). *In vivo* knockdown of S1P1 receptors on dorsal root ganglion neurons leads to reduced pain behaviour after an induced inflammatory episode (Xie et al., 2012). S1P has been shown to promote migration of neural stem cells to the site of injury in spinal cord injury, this occurs in an S1P1 receptor specific manner (Kimura et al., 2007). Neural cells exhibit endogenous SphK activity that is necessary for the local production of active FTY720-P (Billich et al., 2003). Studies have shown that FTY720-P reduces neuronal injury after cerebral ischemia, this is dependent on activation of Akt and ERK second messengers

(Hasegawa et al., 2009). *In vitro* studies have shown that FTY720-P enhances the levels of endogenous brain derived neurotrophic factor (BDNF) in neuronal cultures, in a time and concentration dependent manner (Chun and Hartung, 2010). Due to the lack of affinity of FTY720-P for S1P2 receptors it is possible that the drug could have a positive effect on neuronal function through its action on S1P1 receptors alone. However, although FTY720-P is distributed throughout the brain it does not appear to colocalise with neurons, instead localising to white matter tracts (Foster et al., 2007; Oyama et al., 1998).

1.3.6 S1P receptors in Oligodendrocytes.

The early apoptotic and necrotic loss of oligodendrocytes is a seminal and unexplained feature of MS lesions. The S1P5 receptor, and to a lesser extent the S1P1 receptor appear to be the main S1P receptors expressed in oligodendrocytes, and are expressed throughout development of these cells from oligodendrocytes progenitor cells (OPCs) to mature myelinating oligodendrocytes (Yu et al., 2004). OPCs are important in the context of MS due to their role in remyelination. Remyelination has been observed during the early, mainly inflammatory phases of MS, but seems to fail during the later disease stages (Bruck et al., 2003). It has also been shown that FTY720-P has a direct cytoprotective/anti-apoptotic on OPCs (Coelho et al., 2007). *In vivo* studies on both human derived and rodent derived OPCs show that FTY720-P treatment can rescue these cells from apoptosis induced by growth factor withdrawal and pro-inflammatory cytokine treatment (Coelho et al., 2007; Miron et al., 2008a). Remyelination requires the proliferation and migration of OPCs to areas of demyelination followed by differentiation into mature oligodendrocytes (Franklin and Ffrench-Constant, 2008). Both FTY720-P and S1P appear to have no effect on OPC proliferation, while both impede cellular migration via the S1P5, $G_{12/13}$, Rho signalling pathway (Novgorodov et al., 2007). Prolonged treatment with higher doses of FTY720-P induces process extension via S1P1 receptor signalling (Antel and Miron, 2008). A recent study has shown that FTY720-P can inhibit LPC-induced demyelination in organotypic cerebellar slices, in an S1P1 receptor specific manner (Sheridan and Dev, 2012). This inhibition of demyelination correlated with inhibition of release of a number of cytokines (Sheridan and Dev, 2012). The S1P5 receptor is expressed in the white matter tracts and high levels of mRNA have been observed in oligodendrocytes (Im et al., 2000). The S1P5 receptor has been shown to be coupled to both G_i and G_{12} and therefore may be important in the seemingly opposing functional effects of FTY720-P (Malek et al., 2001). S1P5 receptor knockout animals show no reduction in myelination; however this may be due to redundancy in the S1P signalling pathways. S1P5 receptor deficient immature oligodendrocytes do show reduced response to S1P (Jaillard et al.,

2005). Some studies show that following demyelination, FTY720-P can significantly enhance markers for remyelination, in particular myelin basic protein (MBP) (Jackson et al., 2011). It is suggested that this effect is due to activation of S1P5 receptors on activated microglia, leading to the reduced production of pro-inflammatory cytokines (Jackson et al., 2011). It is clear that FTY720-P regulates oligodendrocytes in varying capacities; the significance of these findings in relation to Gilenya® remains unclear.

1.3.7 S1P receptors and Endothelial Cells of the Blood Brain Barrier.

The BBB is a natural, physical and metabolic barrier created by tight junctions between the endothelial cells of the CNS vasculature and their close relationship with astrocytic cellular protrusions known as "end-feet" (Paolinelli et al., 2011). The function of the BBB is to separate the circulating blood from the cerebrospinal fluid (CSF) and prevent the transfer of large or hydrophilic molecules into the CNS, while allowing the diffusion of small hydrophobic molecules (Correale and Villa, 2007). The BBB actively transports vital metabolically relevant molecules such as oxygen, glucose and carbon dioxide. The BBB has been shown to be compromised in MS, characterized in particular by abnormalities in the tight junctions between endothelial cells. Tight junction dysfunction is a hallmark of not just MS but of other neuroinflammatory CNS diseases including Alzheimer's disease (McQuaid et al., 2009). A compromised BBB leads to transendothelial migration of lymphocytes and macrophages which is a major step in triggering neuroinflammation (Minagar and Alexander, 2003). Demyelinating inflammatory plaques have been shown to accumulate around small blood vessels (Correale and Villa, 2009; Raine, 1994). Moreover endothelial cells express high levels of S1P1 and S1P3 receptors and can produce and release S1P (Wang and Dudek, 2009) (Brinkmann et al., 2004). Studies have shown that treatment of human pulmonary endothelial cells with S1P reduces permeability in an S1P1 receptor specific manner (Singleton et al., 2006). However, S1P3 receptor activation is associated with Rho signalling and subsequent increased barrier permeability (Singleton et al., 2006). As FTY720-P is a full agonist of S1P1 receptors and only partial agonist of S1P3 receptors, it is possible that S1P1 receptor mediated, enhanced blood brain barrier integrity may contribute to the drugs clinical efficacy by excluding auto-reactive T-cell entry to the CNS.

1.4 Astrocytes

1.4.1 Astrocytes: An Introduction.

Glial cells make up ~90% of the cells found in the human brain, the most abundant of these cells are astrocytes. Traditionally astrocytes were regarded as merely inert scaffolds, offering structural rigidity, but little else in terms of regular brain function. Astrocytes are stellate cells with varying numbers of processes giving them their characteristic star shape (He and Sun, 2007). They are structurally, physically and biochemically diverse which is reflected in their broad spectrum of functions. Astrocytes can be distinguished by their expression of the intermediate filament protein glial fibrillary acidic protein (GFAP), now an established cell-specific marker. Astrocytic functional abnormalities have been heavily implicated in various brain pathologies including glioblastoma, amyotrophic lateral sclerosis, Alexanders disease and MS (Gorospe and Maletkovic, 2006; Miljkovic et al., 2011).

1.4.2 Astrocytic function in the CNS.

It is well established that the initial classification of astrocytes as 'housekeeping' or 'maintenance' cells is a poor reflection on their role and importance in the CNS. Astrocytes have been implicated in a large number of vital processes in the brain; their processes extend into synapses, nodes of Ranvier and are major components of the BBB (Bacci et al., 1999; Paolinelli et al., 2011; Perea and Araque, 2010). Astrocytes are capable of 'sensing' neuronal activity through the wide range of ion channels and neurotransmitter receptors that decorate the plasma membrane of these cells. Astrocytes occupy exclusive territories, within these domains stable associations with the microvasculature, numerous neurons and up to thousands of synapses are possible. Astrocytes also contain vesicles and other molecular machinery necessary for exocytosis, with Ca^{2+} dependent glutamate release observed in cultured astrocytes (Kreft et al., 2004). Glutamate has been found to cause an increase in cytosolic Ca^{2+} in both cultured astrocytes and brain slices (Porter and McCarthy, 1996). This led to the concepts of "gliotransmission" and "non-electric excitation", making astrocytes active elements of the brain circuitry (Parpura and Zorec, 2010). Astrocytes can 'sense', integrate and respond to neuronal signals (Zorec et al., 2012). The best studied of which is the response of astrocytes to glutamate with oscillations in their internal Ca^{2+}, these oscillations can then propagate as waves to neighbouring astrocytes (Charles et al., 1991). Astrocytes receive direct stimulation from a range of neuronal subtypes and it also has been shown that astrocytes display spontaneous internal Ca^{2+} oscillations and cause NMDA receptor mediated neuronal excitation (Parri et al., 2001). This can be

confined to distal regions of astrocytes or can propagate to other cells (Parri et al., 2001). This challenges the established view that astrocytes are purely reactive elements by demonstrating that these cells can indirectly generate neuronal activity in the mammalian CNS. Evoked or spontaneous changes in internal Ca^{2+}, is a form of non-electric excitability and can cause various cellular effects such as activation of protein kinases, vesicular release and activation of ion channels (Takata and Hirase, 2008). In response to elevations in internal Ca^{2+} astrocytes release a wide range of signalling molecules and neuroactive molecules named gliotransmitters. This gliotransmission is mediated by various chemical transmitters including ATP, glutamate, D-serine, taurine, atrial natriuretic factor and TNF-α (Halassa and Haydon, 2010). Astrocytes play a key role in formation of synapses or synaptogenisis. Synaptogenisis occurs throughout adult life, but is highly active during early brain development. Synaptogenisis coincides with the appearance of astrocytes in the CNS (Wang and Bordey, 2008). Once thought to provide structural support to synapses, recent studies have shown astrocytes to be active participants in synaptic transmission (Oliet et al., 2001). Co-culturing of pure neurons with astrocytes causes a huge increase in the number of structurally mature and functioning synapses (He and Sun, 2007). These data indicate that astrocytes induce the formation of synapses by releasing various synaptogenic signals that are importfant in the development, maturation and maintenance of synapses. Some identified signals include thrombospondins (a small family of secreted glycoproteins), and cholesterol in a complex with the lipid transporter apolipoptotein A (Allen and Barres, 2005; Barres and Smith, 2001). Neuronal differentiation, maturation, survival and neurite outgrowth are processes that are all influenced by the release of various growth factors from astrocytes (Ransom and Ransom, 2012). It is well established that astrocytes release many growth factors *in vitro* including nerve growth factor (NGF), neurotrophin-3, fibroblast growth factor (FGF) and brain derived neurotrophic factor (BDNF) (Ransom and Ransom, 2012). Consequently there are a myriad of implications consequent to dysfunction of these processes with respect to disease pathogenesis.

1.4.3 Astrocytes in Multiple Sclerosis.

Astrocytes become activated in a process known as 'reactive gliosis' in response to a pathological insult (Nair et al., 2008). The formation of the glial scar is an important process undertaken by astrocytes following CNS tissue injury (Correale and Villa, 2007). Reactive astrocytes have an altered phenotype compared to the normal functioning quiescent astrocytes. It is now believed that altering astrocytic behaviour can be therapeutically beneficial in treating numerous CNS disorders. Extensive astrocytic proliferation is accompanied by wholesale changes in adhesion molecule, growth factor,

cytokine and antigen presentation molecule expression. Reactive astrocytes are the main constitutes of the demyelinating plaques found in MS (Wekerle, 2008). Similar to scarring found elsewhere in the body the purpose of the glial scar is to protect from further damage and to initiate the healing process (Zeinstra et al., 2003). However, the existence of such a scar has its disadvantages by presenting a physical and chemical barrier to axonal regrowth. It also hides the inflammatory and demyelinating plaques from circulating pharmacological agents (Correale and Villa, 2009). In the context of MS plaques, astrocytes have been observed to express high levels of nitric oxide synthase (Hill et al., 2004). Nitric oxide and superoxide radicals' production may directly or indirectly damage oligodendrocytes, myelin and/or axons. These reactive oxygen species activate $NF_{K}B$ dependent signalling processes. This in turn induces expression of pro-inflammatory molecules matrix metalloproteinase and inducible nitric oxide thereby having a detrimental effect on barrier integrity (Smith and Lassmann, 2002). Another major role for astrocytes is as part of the natural barrier between the circulating blood and the 'internal milieu' of the CNS. Astrocytes are essential for maintaining a robust BBB phenotype; with loss of contact between astrocytes and the cerebral endothelial cells abolishing the barrier. Astrocytes appear to provide structural and trophic support to the large quantities of cerebral endothelial cells which are connected by tight junctions (Ballabh et al., 2004; Correale and Villa, 2009). Activation of the epidermal growth factor receptor (EGFR) on astrocytes following neuronal injury causes quiescent astrocytes to become reactive. This activation leads to up-regulation of genes related to migration, matrix metalloproteinase-3/16/24, laminin, collagens XI, XV, and XVIII, fibronectin and chondroitin sulfate proteoglycans. Activation of the EGFR pathway also leads to the release of leukocyte chemoattractants and the synthesis of inducible nitric oxide (iNOS) (Liu et al., 2006; Liu and Neufeld, 2007). Astrocytes cannot form myelin; however these cells can promote myelination (Nash et al., 2011). It has been shown that activity dependent release of ATP from neurons can signal to astrocytes via the purinergic P2 receptors (Franke et al., 2012). This purinergic signalling causes astrocytic release of the cytokine leukaemia inhibitory factor (LIF) which is shown to promote myelination by mature oligodendrocytes (Ishibashi et al., 2006). Therefore it is possible that LIF released by the reactive astrocytic element of MS inflammatory lesions could promote remyelination. However it has also been shown that under certain pathological conditions high levels of LIF can inhibit both myelination and remyelination. The link between astrocytes and myelination is not new. Alexander disease is a disease of the white matter of the CNS caused by mutations in the GFAP gene (Gorospe and Maletkovic, 2006). It is plausible that excess release of anti-myelination factors caused by an atypical astrocytic phenotype could disrupt this vital process (Li et al., 2002).

The fact that all S1P receptors except S1P4 receptors are ubiquitously expressed in the cells of the CNS (Malchinkhuu et al., 2003) coupled with the distribution of FTY720-P to the brain due to its lipophilic properties point towards a possible role for FTY720-P in the CNS. While functional antagonism of S1P1 receptors on pro-inflammatory, autoreactive T-cells in the periphery appears to be the primary mode action of FTY720-P; evidence also links FTY720-P with a more CNS neuroprotective role. Functional antagonism of S1P1 receptors on astrocytes may dampen the negative effects of astrogliosis in the MS brain and also improve gap junctional communication between the cellular components of BBB. Investigating the CNS effects of FTY720-P on astrocytes via modulation of the S1P1 receptor is the primary research aim of this thesis.

1.5 Aims and Hypothesis.

Indications are that astrocyte expressed S1P1 receptors may be involved in propagating the neuroinflammatory aspect of EAE and thus MS (Choi et al., 2011; Kang et al., 2010). The aim of this thesis is to further examine the role of S1P1 receptors in astrocytes and to investigate the effect of receptor localisation on receptor function. Specifically we aim to investigate the effect of disruption of putative c-terminal interactions on S1P1 receptor localisation. In addition to this we aim to analyse various downstream signalling pathways and again examine how blockage of c-terminal protein-protein interactions may affect this signalling. Finally we hope to provide evidence that astrocytes can contribute to a neuroinflammatory environment through the release of pro-inflammatory cytokines, and show that S1P receptor modulation may have inhibitory effect on this. In this way we aim to investigate if S1P receptors expressed on astrocytes are a legitimate molecular target for the MS therapy, Gilenya®.

We hypothesise that S1P1 receptor localisation may be altered through the inhibition of putative protein-protein interactions at the receptors c-terminus. In addition we hypothesise that S1P receptor signalling may be involved in the release of pro-inflammatory signals from astrocytes and that pharmacological modulation of these receptors may have an anti-inflammatory effect through inhibition of these signals.

Chapter 2. General Material and Methods

2.1 Culture of primary cells.

2.1.1 Aseptic technique.

For all *in vitro* cell culture experiments and procedures aseptic technique was strictly adhered to. All pipette tips, non-sterile plastic consumables and dissection kits were autoclaved and sprayed with 70% alcohol before being placed in the laminar flow hood (Mason Technologies, Ireland). The interior of the hood including all equipment was exposed to a germicidal UV lamp which emits ultraviolet radiation at 253.7nm. This final step ensures complete sterility. A cell culture laboratory coat was worn at all times as were disposable protein free gloves which were sprayed with 70% alcohol upon entry back into the laminar flow hood

2.1.2 Tissue culture flask and coverslip preparation.

Poly-L-lysine was utilised as an attachment factor utilised to improve cell adherence. A poly-L-lysine solution (40µg/ml in sterile H_2O: Sigma Aldrich, UK) was prepared. This solution was added to each culture flask and left for 1hr at 37°C. The solution was aspirated and the flasks washed twice with cell culture grade H_2O (Sigma Aldrich, UK) and left to dry thoroughly in the laminar flow hood. For growing cells on coverslips, borosilicate glass coverslips with a diameter of 13mm (VWR Ireland) were sterilized prior to use by immersion in 70% methanol followed by exposure to UV light overnight. Coverslips were then coated with poly-L-lysine (40µg/ml) for 1hr at 37°C. The coverslips were subsequently laid out in the laminar flow hood to air dry before being placed in 24 well-plates (Sarstedt, Germany). The plates were stored for up to 2 weeks at +4°C.

2.1.3 Preparation of mixed cortical glia.

Primary cortical mixed glial cultures were prepared using postnatal one-day old female Wistar rats (supplied by the Bioresources Unit, Trinity College Dublin). Under sterile condition in a laminar flow hood (FASTER, Ultrasafe Class II Microbiological Safety Cabinet) the pups were decapitated in accordance The Animals Act 1986 (Scientific Procedures) Schedule I guidelines. The skull was exposed by cutting the skin from the neck down to the tip of the nose. The skull was removed by making a sagittal cut along the level of the medial longitudinal fissure and two horizontal cuts along each side of the skull at the level of the ears. The skull was peeled back revealing the cortex which was rapidly dissected out. The cortices were placed in warmed Dulbecco's Modified Eagle Medium F12 (Biosera, UK) which was supplemented with 10% heat inactivated foetal bovine serum (Biosera, UK) and 1% penicillin/streptomycin (Gibco BRL, Ireland). The meninges were removed from the

cortices using a fine straight forceps and the cortices were then cross chopped using a sterile disposable scalpel. The tissue was incubated in warmed supplemented DMEM/F12 for 20mins at 37°C. Following incubation the tissue was triturated and passed through a sterile nylon mesh cell strainer (40µm; BD Biosciences, USA). This filtrate was centrifuged at 2,000g for 3mins at room temperature. The resulting cell pellet was resuspened in pre-warmed supplemented DMEM/F12 and a cell count was carried out using a tryphan blue exclusion method. The resuspened cells were plated at a density of 1 x 10^6 cells/ml on poly-L-lysine coated coverslips in 24-well plates. The cells were allowed to adhere for 3hr after which the wells were flooded with 500µl pre-warmed supplemented DMEM/F12. Primary cells were maintained in a humidified incubator supplied with 5% CO_2 and 95% air, set at a temperature of 37°C. The media was changed the day following the initial preparation and every 3 days thereafter. Mixed cortical glial cultures and pure astrocyte cultures were maintained in supplemented DMEM/F12. Cells were grown for 10 days or until confluent before treatment.

2.1.4 Preparation of pure astrocyte cultures.

Glial cultures were prepared using postnatal one-day old female Wistar rats (supplied by the Bioresources Unit, Trinity College Dublin). The cortices were dissected out as above and placed in supplemented DMEM/F12. The tissue was incubated in this media for 20mins at 37°C. Following incubation the tissue was triturated and passed through a sterile nylon mesh cell strainer (40µm; BD Biosciences, USA). This filtrate was centrifuged at 2,000g for 3min at room temperature. The pellet was resuspended in an appropriate amount of pre-warmed DMEM/F12. The resuspened cells were plated on poly-L-lysine coated T75 culture flasks (Sarstedt, Germany), usually two cortices per T75 flask. When confluent (~13 days) the flasks were removed from the incubator, the neck and caps were tightened and the flasks made air-tight with parafilm. The flasks were then shaken for 3hr at 37°C and at 200 RPM in an orbital shaker (New Brunswick Scientific, Excella E24). Each flask was then tapped ~30 times and the media poured to remove the microglia. The remaining flasks were incubated with 2mls trypsin-EDTA (Sigma Aldrich, UK) for 10mins at 37°C. Supplemented media was added to the flasks to inhibit the trypsin and the flasks were again tapped, the resulting cell suspension was collected and centrifuged at 2,000g for 3mins at room temperature. This pellet was resuspended in 8mls DMEM/F12, a cell count was carried out and the cells were plated at a density of 1 x 10^5 cells/ml on poly-L-lysine coated glass coverslips in 24-well plates. The cells were allowed to adhere for 3hr after which the wells were flooded with 500µl pre-warmed supplemented DMEM/F12. Cells were maintained as in previous section.

2.1.5 Preparation of human astrocytes.

Primary human astrocytes, derived from post-mortem cerebral cortex embryonic tissue were purchased from the provider (Sceincell, USA, HA1800). Cryopreserved cells were thawed in a 37°C waterbath and transferred to a poly-L-lysine coated culture flask. Cells were maintained in the manufacturers astrocyte specific media (HA1801) supplemented with 10ml fetal bovine serum (0010), 5ml astrocyte growth supplement (1852) and 5ml penicillin/streptomycin (0503). Human astrocytes were grown until they reached 90% confluency where the cells subcultured or seeded to 24-well plates as previously described (see section 2.1.4).

2.1.6 Organotypic slice culture preparation.

Experiments were conducted using tissue isolated from postnatal day 10 (P10) Wistar rats of either sex. The cerebellar slice culture preparation was based on published protocols (Birgbauer et al., 2004). Briefly, the brains were removed and placed in ice cold serum supplemented media, referred to as organo-serum media (50% Opti-Mem, 25% Hank's Buffered Salt Solution, 25% Heat inactivated horse serum, 1.1% D-glucose {27.5mM}, 1.1% Glutamax {2mM}, 1% Penicillin {10,000U/ml} and Streptomycin {100U/ml} (Pen/Strep) and 1% HEPES {10mM}). After 1-2 minutes the cerebellum quickly dissected out. 400μm parasagittal slices of cerebellum were cut using a McIlwain tissue chopper. Three slices were grown on 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*, slices were grown in organo-serum media. After 3 days, slices were transferred to serum-free medium (98% Neurobasal-A and 2% B-27 {Invitrogen}, supplemented with 2mM Glutamax, 28mM D-glucose, 100U/mL penicillin/streptomycin and 25mM HEPES). Slices were cultured for up to 14 days.

2.1.7 Trans-Activator of Transcription, "TAT" Protein.

Delivery of therapeutics for the treatment of neurodegenerative diseases remains an important hurdle for pharmaceutical companies in the search for new cerebral acting compounds. Therapies that have intracellular targets need to be sufficiently polar to allow ease of administration, they need to able to transverse the BBB, and be sufficiently lipophilic to allow access to the cell across its lipid bilayer. Some compounds need to be modified quite drastically to alter their physical properties which in turn can affect their therapeutic value. One way of delivering small peptides, drugs and proteins across the BBB is to fuse the protein/compound of interest with a cell penetrating protein such as the antennapedia peptide or the HIV-TAT protein (Console et al., 2003). HIV-1 encodes a small regulatory protein known as trans-activator of transcription (TAT) (Cai et al., 2011). This ~10

kDa protein is a transcriptional activator which can bind both HIV DNA and RNA and host cell DNA. After binding to the HIV transactivating region, TAT induces a several fold increase in HIV transcription. HIV-TAT can be released from the host cell, cross the cell membranes of near-by cells and has been shown to promote the expression of inflammatory genes such as iL-6 and TNF-β and repress MHC-I presentation, CD25 and IL-2 expression (Johri et al., 2011). Various theories exist as to mechanism by which TAT protein crosses cell membranes; these include caveolar endocytosis, clathrin dependent endocytosis and macropinocytosis (Campbell and Loret, 2009). However the exact cellular mechanisms involved in TAT uptake and internalisation are still unknown. While in theory the use of cell penetrating peptides such as TAT protein to mediate the delivery of compounds to the CNS has many advantages it also has several caveats. The main disadvantage of using TAT protein *in vivo* is the systems lack of selectivity whereby every cell that the protein comes in contact with will be transduced by the TAT fusion protein (Dietz and Bahr, 2004). However the use of the TAT protein provides a highly reliable and useful tool for transducing cultured cells with a protein of interest in an *in vitro* setting. We take advantage of the infectious properties of the TAT protein to allow us to study the effects of small peptides on the intracellular trafficking and signalling of specific GPCRs, in this case the S1P1 receptor.

2.2 Biochemistry

2.2.1 SDS Poly-Acrylamide-Gel-Electrophoresis.

SDS-PAGE was utilised to separate proteins according to their molecular weight. The 10% SDS (sodium dodecyl sulphate) (Sigma Aldrich, UK) disrupts the non-covalent bonds in the proteins thereby denaturing the secondary and tertiary structures. It also imparts a negative charge on the protein that is proportional to its mass. Tetramethylethylenediamine and ammonium persulfate (TEMED and APS; Sigma Aldrich, UK) were used to catalyze the polymerization of acrylamide in the formation of the gel. Tris buffer (0.4mM Tris-HCL; pH 8.8) was utilised to buffer the preparation. The 'pore' size of the gel is determined by the ratio of acrylamide to bis-acrylamide, 30% acrylamide/bis-acrylamide were utilised. The casting stand, glass plates and combs were all washed with dH_20 and methanol, followed by assembly of the apparatus (Bio-Rad laboratories, UK). A 10% separating gel was prepared as follows (0.375M Tris, 0.1%SDS, 10% acrylamide, 0.1%APS and TEMED). Approximately 6.5ml of the separating gel was added to the plates followed by 1ml water saturated butan-1-ol which prevents the gel from drying as it sets. This was left for 60mins at room

temperature to set. A 4% stacking gel was prepared as follows (0.125M Tris, 4% Acr/Bis, 0.1% SDS, 0.1% APS and TEMED). The butan-1-ol was removed and the gel washed with 3 x 1ml dH_2O. The stacking gel was added to the set separating gel, the comb was introduced and it was left set for a further 60mins. To denature the samples (see section 2.2.3) 2 x sample buffer (Bio-Rad laboratories, UK) was added. The sample buffer contains bromophenol blue a colour marker, and is supplemented with 0.5% β-mercaptoethanol which reduces disulphide bridges in proteins so that they can adopt a linear configuration necessary for separation by size. The samples are heated in a water bath for 5mins at 95˚C; they were then cooled to room temperature. The samples were centrifuged at 12,000 g for 5mins which removes insoluble material to prevent streaking. The gasket was flooded with 1x running buffer (25mM Tris-base, 0.192M glycine, 0.1% SDS). A pre-stained molecular weight marker (3µl; Bio-Rad laboratories, UK) was introduced to the first well, followed by 20µl of sample in the remaining wells. The gel was run at a constant voltage of 100v for 15mins until the samples had diffused out of the separating gel, it was then run at 200v for 60mins. Migration of the bromophenol blue was monitored and the power supply switched off before the samples ran from the gel. A gel stain and/or Western blot was then performed as required.

2.2.2 Western Blot.

The PVDF (Polyvinylindene fluoride; Immobolin P, Micropore) membrane was activated by immersion in 100% methanol for 10secs followed by immersion in solution C (25mM Tris, 0.02% SDS, 20% methanol and 40mM ε-amino-capronic acid) for 60mins. Two filters (Whatman filters, Grade 3) were immersed in solution A (0.3M Tris, 0.02% SDS, 20% methanol), solution B (25mM Tris, 0.02% SDS and 20% methanol) and solution C respectively just before use. The blot was run at 100v for 90mins allowing the transfer from the gel to membrane. The membrane was blocked overnight in 5% bovine serum albumin (BSA; Sigma Aldrich, UK) and dry skimmed milk (Marvel) in PBS-T (100mM Sodium chloride, 80mM Sodium phosphate, 20mM Sodium dihydrophosphate and 10% Tween 20) to prevent non-specific binding. Primary anti-S1P1 receptor or anti-GFAP antibody (see table 2.1) (2.5µl antibody in 10ml PBS-T and milk) were incubated with the membrane for 1hr. The membrane was then washed 3 x 5mins with PBS-T. The membrane was then incubated with HRP conjugated anti-rabbit or anti-mouse secondary antibodies (see table 2.1) (5µl antibody in 10ml PBS-T and milk) for 1hr. The membrane was subsequently washed 3 x 5mins in PBS-T to eliminate any unbound antibody. Visualisation of the bands was carried out using Immobolin chemiluminescent HRP substrate (Millipore, WBKLS0500). Blots were imaged on a Fujifilm LAS-3000 Intelligent Dark-box.

2.2.3 Cell preparation for Western blotting.

Treated cultures in 24-well plates were scraped in sterile PBS and collected in a sterile 1ml eppendorf tube. Cells were centrifuged at 12,000 g for 5mins to pellet the cells. Cell pellets were resuspended in varying amounts of PTxE buffer (PBS, 1% triton-x-100 and 0.1mM EDTA), depending on pellet size. This suspension was then stored at -20°C or -80°C for long term storage. Samples were thawed on ice and diluted 1:1 in sample buffer. Samples were heated to 100°C in a heating block for 5mins and then allowed to come to room temperature. The samples were then centrifuged at 12,000g for 5mins to remove insoluble material which would cause streaking, and subjected to SDS-PAGE and Western blotting.

2.2.4 Adenylate cyclase assay

Astrocytes were trypsinized, diluted and seeded into 24-well cell culture plates (MidSci, USA) at a cell density of 5 x 10^5 cells/ml. Cells were left for 2-3 days before assaying for cAMP levels, as previously described (Salomon, 1979). Briefly, cells were incubated in 500 µl of serum free DMEM/F12 for 3hr at 37°C/5% CO_2 before use. Cells were then loaded with [H^3] adenine in serum free media for 4hr at 37°C/5% CO_2 and then washed with PBS supplemented with $CaCl_2$ (0.9mM) and $MgCl_2$ (0.5mM). Stimulations were carried out in Hank's balanced salt solution (HBSS; Invitrogen, USA) with 20µM forskolin (Sigma Aldrich, Germany). Stimulation were carried out in the presence of the phosphodiesterase inhibitors 1mM IBMX (3-isobutyl-1-methylxanthine; Enzo Life Sciences, USA), 10µM rolipram (Enzo Life Sciences, USA) and 1µM BAY 60-7550 (Enzo Life Sciences, USA) to inhibit degradation of cAMP. Cells were treated either with 1µM FTY720-P for 20mins to determine immediate effects on cAMP levels, or treated with 1µM FTY720-P for 1hr followed by a 5hr washout period to determine effects on persistent signalling. Cells were also pre-incubated with or without 100µg/ml MNP301 for 1hr, prior to addition of FTY720-P.

2.2.5 Measurement of cytokine/chemokine release from astrocytes by ELISA

Cells were brought to ~80% confluencey and starved in serum-free media for 4hr before stimulation. Astrocytes were stimulated with a range of both recombinant human IL-17 (5ng/ml-500ng/ml) alone and in combination with recombinant human TNF-α (1ng/ml-100ng/ml) (gift from Dr. Anis Mir, Novartis Pharma, Switzerland) or with 100ng/ml lipopolysaccharide (LPS) (Enzo Life Sciences: 581-007-L002). Stimulation with cytokines was carried overnight for 18hr. Supernatant was then removed from the cells and frozen at -20°C for analysis by ELISA (R and D Systems, USA). 96-well ELISA plates (NUNC, Thermo Scientific, Denmark) were coated overnight at room temperature with

anti-IL6 capture antibody diluted in PBS (**Fig.** 2.1). The plates were washed three times with wash buffer (0.05% Tween 20, PBS, pH 7.4) and then blocked for 2hr at room temperature with reagent diluent (1% BSA, PBS, pH 7.4). The plates were then washed three times with wash buffer and any remaining buffer was removed from the wells by aspiration. A standard curve was prepared using serial dilutions of the recombinant protein diluted in reagent diluent (1% BSA, PBS, pH 7.4). All points of the standard curve were duplicated. 100µl of samples were loaded into appropriate wells in triplicate. The samples and standard were incubated in the antibody coated ELISA plate for 2hr at room temperature. The plate was then washed three times with wash buffer and 100µl of detection antibody (**Fig.** 2.1) diluted in reagent diluent was added to all wells. Following three further washes, 100µl of streptavidin-HRP diluted in reagent diluent was added to each well and incubated for 20mins at room temperature, protected from light. Three further washes were carried out and the wells were incubated with 100µl substrate solution (TMB; R and D Systems, UK) for 20mins at room temperature, protected from light. The colour reaction was stopped by the addition of 50µl 1M H_2SO_4 and absorbance was read immediately using a plate reader at 450nm (Labsystem Multiskan RC, UK). The standard curve was calculated by plotting the standards against the absorbance values (GraphPad Prism version 4; GraphPad software, USA), LIX and IL-6 levels were measured in pg/ml.

2.2.6 Calcium signalling.

Cells were grown until ~80% confluencey on poly-d-lysine coated, glass bottomed, 35mm FluoroDishes (World Precision Instruments, USA). Cells were pre-treated with either 1µM S1P, AUY954 or FTY720-P in serum free DMEM/F12 for 1hr. Cells were then washed 2x serum free media and left in fresh serum free media for 3hr. Cells were washed once with 1ml 37°C HBSS (Invitrogen, USA) supplemented with 20mM HEPES buffer (Invitrogen, USA) and 20mM glucose (Sigma Aldrich, Germany). Cells were loaded with 2µM Fura-4 AM (Invitrogen, USA) in supplemented 37°C HBSS for 20mins at 37°C and 5% CO_2. Fura-4 AM dye was removed and cells were washed once with 37°C supplemented HBSS. Next, cells were left to rest in 1ml supplemented HBSS at room temperature in the dark for 20mins. Calcium responses were recorded using a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss Ltd, UK), scanning speed was 1 frame/sec. Stimulation of cells was performed by adding a 3x concentrated solution of test reagent (3µM S1P/AUY954/FTY710-P) in supplemented HBSS with a manual pipette. Baseline recordings were taken for 30secs, test reagents were then added and changes in $_iCa^{2+}$ levels were recorded for a further 150secs. After 180secs, 1µM glutamate (Sigma Aldrich, Germany) was added and recording was continued for a further 60secs. Recording was terminated at 240secs.

2.2.7 Dot blot arrays.

Conditioned rat astrocyte supernatants were analysed for relative increases or decreases in a range of 29 cytokines and chemokine using a rat cytokine array dot blot system (R and D systems, ARY008). Cell supernatants were incubated on nitrocellulose membranes which have an array of capture antibodies spotted on them in duplicate. Supernatants (1ml) were incubated with 15µl detection antibody cocktail (reconstituted with 100µl deionized H_20). Membranes were incubated with antibody and supernatant mix overnight at +4°C. Membranes were washed on a rocking platform shaker with 'Wash Buffer' three times to remove unbound material. Streptavidin-HRP (2ml) was added to each washed membrane for 30mins at room temperature. Wash steps were subsequently repeated and blots were incubated with Immobilon Western chemiluminescent-HRP substrate (Millipore, WBKL20050) for 20mins in the dark. Cytokine antibody arrays were imaged using the FujiFilm LAS-3000 imaging system. Images were captured as 8 bit grayscale tifs and the relative pixel density of each spot on the array was quantified using Image J software.

2.3 Immunocytochemistry

2.3.1 Compound treatment of primary cells.

Supplemented media was removed from the wells, 500µl of serum free DMEM/F12 was added to each well. Cells were incubated in the serum-free media for 3hr prior to treatment. Negative controls were treated with DMSO for 1hr at 37°C. In the main, compound treatment was at 37°C for 1hr using a 1µM concentration of compound. Specific treatment details are indicated in the figure legends. After compound treatment cells were subjected to immunocytochemistry

2.3.2 Whole cell immunostaining for primary cultures.

Post-treatment, cells were washed in warm PBS (Sigma Aldrich, UK) followed by fixation in ice-cold 100% methanol for 10mins. Cells were washed 3 x 5mins in sterile PBS. The cells were permeabilised by incubation with 0.2% Triton-X-100 (Sigma Aldrich, UK) in PBS for 5mins at room temperature. Non-reactive sites were blocked overnight at +4°C with blocking buffer which consisted of 10% normal goat serum (Gibco BRL, Ireland) and 2% bovine serum albumin (Sigma Aldrich, UK) in PBS. The following day the blocking buffer was removed and the cells incubated in primary antibody (see **Fig.** 2.1) overnight at 4°C. The primary antibody was then removed and the cells washed 3 x 5mins PBS. Secondary fluorescent antibody (see **Fig.** 2.1) was applied to the cells for 2hr at room

temperature and kept in the dark to prevent photo bleaching. The coverslips were then washed 5 x 5mins in PBS and removed from their wells, dried and mounted on a microscope slide in Vectashield® Mounting Medium (Vector Laboratories, USA). The edges of the coverslip were sealed with nail varnish and stored at +4°C until required. Working dilutions of antibodies used was determined prior to use. For antibodies and dilutions utilised (**Fig.** 2.1). The cells were then imaged using a Zeiss LSM 510 META confocal laser scanning microscope utilising an Axiovert 200M inverted microscope (Zeiss Ltd, UK). Images were acquired and optimized using LSM 510 computer program and were acquired at a 63x magnification unless otherwise stated. Initially optimal settings for image quality were obtained and thereafter the settings remained unchanged.

2.3.3 Image analysis and quantification of receptor internalisation

Image analysis was carried out using the bioconductor package, EBImage (http://www.bioconductor.org/help/bioc-views/release/bioc/html/EBImage.html), for the R statistical programming environment. Briefly, red, green and blue channels were separated for each image and every pixel within the images (1024 x 1024) was assigned an intensity value between 0 and 1. The blue channel represents Hoechst-stained nuclei and the red channel GFAP-labeled astrocytic processes. Both channels were used to label the nuclear, perinuclear and cytoplasmic compartments of the astrocyte cells (**Fig.** 2.2). An analysis script was written in R to measure the fluorescence intensity of S1P1 receptor staining (i.e. green channel) in each of these 3 distinct cellular compartments in all cells within the confocal images. Using the blue (Hoechst) channel, minimum size and fluorescence intensity thresholds were set in order to select only those pixels that belong to Hoechst-labeled nuclei. The nuclei were then 'dilated' using specified morphological kernel expansion. This step allowed the designation of a perinuclear region surrounding each nucleus. The nuclear and perinuclear compartments of each cell were then subtracted from the red channel and the remaining GFAP-labeled processes were used to calculate the average fluorescence intensity of S1P1 receptors in the cytoplasmic compartment of astrocytes within each image. A distance map was then generated for the image which calculates the distance each pixel is from an edge pixel. The watershed segmentation algorithm accurately separates nuclei that are very close together or touching. The perinuclear-to-cytoplasmic fluorescence intensities of each cell were calculated according to the equation [Fp/Fc], where Fp = average perinuclear fluorescence and Fc = average cytoplasmic fluorescence. Increases in the Fp:Fc ratio measures increased S1P1 receptor internalisation and trafficking of the receptor from astroctytic processes to the perinuclear compartment of the cell.

2.3.4 Calcium signalling analysis.

Imaging was carried out using a Zeiss LSM 510 Confocal Microscope with a x40/1.30 oil immersion lens. Images were obtained at a rate of 1 frame/sec for a total of 240secs, at a 512 x 512 pixel resolution and exported as .tiff files. Images were then analysed using the EBImage package as described above. Briefly a threshold pixel intensity value was determined, to reduce the risk of analysis of background staining. Each pixel was assigned an intensity value ranging from 0 to 1 and a fluorescence value was obtained per frame for each cell in the image. Mean baseline fluorescence (0-30secs) was obtained for each cell. Cell fluorescence was normalised to baseline fluorescence (DF/F0). Each image was then subjected to the following exclusion criteria to eliminate non-responsive or over-responsive cells. Cells were eliminated if they failed all three of the following criteria.

1) Sum fluorescence: <300 or >50,000
2) Max fluorescence (test reagent, 30-60secs) : <2 or >1,000
3) Max fluorescence (glutamate, 180-240secs) : <2 or >1,000

Following sorting, the square root of each value was obtained (√ DF/F0). The mean fluorescence, standard deviation (SD) and standard error of the mean (SEM) was then calculated for each frame. The mean, SEM and cell number (n) were subsequently used in GraphPad Prism 4 software to generate calcium response traces presented as √ DF/F0 over time.

2.3.5 Statistical analysis.

All statistical analysis was performed using the GraphPad Prism version 4 statisical package for Windows. Data was expressed as mean +/- SEM. For comparisons between treatment groups both one-way and two-way analysis of variance (ANOVA) were utilised to confirm statistical significance within experiments, appropriate post-hoc tests were performed to analyse differences between individual groups. Student t-tests were also performed as indicated.

Primary Antibody	Host	Use	Manufacturer	Working Dilution
S1P1	Rabbit	ICH/WB	SantaCruz: *sc-25489*	1:500
GFAP	Mouse	ICH/WB	Millipore: *MAB-360*	1:500
LIX	Mouse	ELISA	R and D Systems: *840795*	1:180
IL-6	Mouse	ELISA	R and D Systems: *840113*	1:180
Neurofilament H	Mouse	ICH	Millipore: MAB5262	1:800
CD11b	Rabbit	ICH	Millipore: *MABT149*	1:500
CNPase	Mouse	ICH	Millipore: *MAB326*	1:500
P230	Mouse	ICH	BD Biosciences: BD611280	1:500
LAMP1	Mouse	ICH	BD Biosciences: BD555798	1:500
EEA-1	Mouse	ICH	BD Biosciences: BD610457	1:500
GM130	Mouse	ICH	BD Biosciences: BD610823	1:500

Secondary Antibody	Host	Use	Manufacturer	Working Dilution
Biotinylated anti-Rabbit	Goat	ICH	Vector: BA-1000	1:500
Biotinylated anti-Rat	Goat	ELISA	R and D Systems: 840796	1:240
Biotinylated anti-Human	Goat	ELISA	R and D Systems: 840114	1:240
Avidin-Alexa 488	n/a	ICH	Invitrogen: *S-11223*	1:1000
Avidin-Alexa 633	n/a	ICH	Invitrogen: *S-21375*	1:1000
Anti-Mouse Alexa 633	Goat	ICH	Invitrogen: *A-21052*	1:500
Anti-Rabbit Alexa 633	Goat	ICH	Invitrogen: *A-21071*	1:500
Anti-Goat Alexa 633	Donkey	ICH	Invitrogen: *A-21082*	1:500
Anti-Mouse Alexa 488	Goat	ICH	Invitrogen: *A10667*	1:500
Streptavidin-HRP	n/a	WB	R and D Systems: 890803	1:200

Figure 2.1 *Primary and secondary antibodies utilised.*

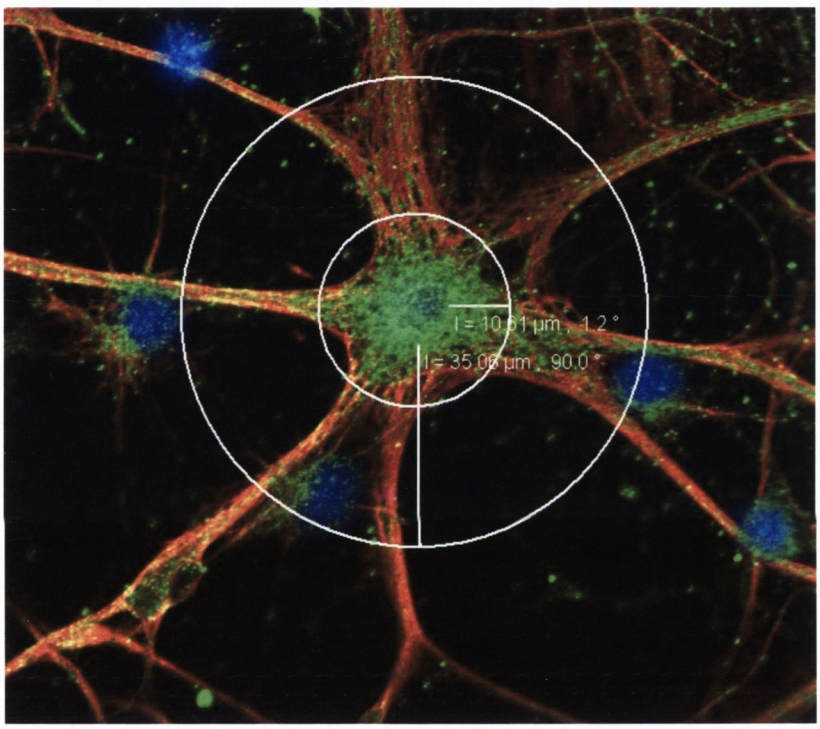


Figure 2.2 *Calculation of receptor internalisation.*

Mean fluorescent measurements were taken from two separate cellular locations. The "nuclear zone" was defined as any positive GFAP staining that was < 10µm from the nucleus. The "process zone" was defined as any positive GFAP staining that was > 35µm from the nucleus. Scale bars, 20µm.

Chapter 3. The Study and Modulation of FTY720-P Induced S1P1 Receptor Internalisation.

1: To establish a protocol for the production of a pure population of primary rat astrocytes.

2: To confirm the expression of the S1P1 receptor in primary rat astrocytes.

3: To investigate the temporal effect of FTY720-P treatment on S1P1 receptor internalisation of astrocytic expressed S1P1 receptors.

4: To analyse the effect of varying concentrations of FTY720-P on S1P1 receptor internalisation.

5: To investigate if FTY720-P induces a prolonged internalisation state in astrocytic S1P1 receptors.

6: To identify the compartment where S1P1 receptors traffic to in response to FTY720-P activation.

7: To analyse the entry of the S1P1 receptor blocking peptide MNP301 into primary rat astrocytes.

8: To investigate the effect of MNP301 on FTY720-P induced S1P1 receptor internalisation in primary rat astrocytes.

Abstract

The sphingosine 1-phosphate receptor subtype 1 (S1P1 receptor) is modulated by the drug Gilenya®, which has been approved as an oral therapy for Multiple Sclerosis (MS). The active ingredient of Gilenya®, namely fingolimod (FTY720), is phosphorylated (FTY720-P) *in vivo* and acts as an S1P receptor agonist. This drug acts on S1P1 receptors on lymphocytes, causing receptor internalisation and down regulation, thereby preventing S1P-mediated egress from secondary lymph nodes and limiting autoimmune response. Importantly, FTY720-P also enters the central nervous system (CNS) where it likely activates S1P receptors in neurons and glia. We have shown previously that S1P receptors promote astrocyte migration and increase mature oligodendrocyte. Here, we report that FTY720-P and the S1P1 receptor selective agonist, SEW2871, but not the natural ligand S1P, internalise S1P1 receptors in rat astrocytes in a concentration- and time-dependent manner. We find the internalised S1P1 receptors localised to the trans-Golgi-network (TGN), where they remain internalised for long periods of time (persistent internalisation). We hypothesised that the extreme carboxy terminus of the S1P1 receptor interacts with trafficking proteins that regulate receptor cycling and designed a novel biologic comprising the last 10 amino acids of the c-terminus of the S1P1 receptor (MNP301) to competitively block these putative interactions. Here, we report that MNP301 prevents FTY720-P mediated persistent internalisation of S1P1 receptors to the TGN. The results show that FTY720-P alters S1P1 receptor surface expression in astrocytes in a manner similar to that reported in lymphocytes and endothelial cells. Collectively, these findings provide further evidence that Gilenya® may provide protection in MS patients through an additional CNS mechanism-of-action, namely astrocytic expressed S1P receptors.

3.1 Introduction

3.1.1 The structure of the S1P1 receptor.

The S1P1 receptor is a traditional 7 transmembrane G-protein coupled receptor. S1P1 contains 3 extracellular loops (Excl), these are ExcL1 between helices 2 and 3, ExcL2 between helices 4 and 5 and ExcL3 between helices 6 and 7. ExcL1 and 2 along with the N-terminal helix create a barrier which limits ligand access to the receptor binding pocket. It is postulated that the most likely point of access for ligands to the binding pocket is within the plasma membrane through a gap created by the 7 transmembrane helices (Hanson et al., 2012). Agonists of the S1P1 receptor can be split into two categories with respect to their interaction with the GPCR. Class I ligands such as FTY720-P are mimics of the natural ligand S1P, they are generally lipid-like in structure, containing structures similar to S1P's sphingolipid head group. Some Class I ligands such as SEW2871 can be non-lipid-like but still contain polar head groups capable of interacting with the S1P head-group region of the receptor. Class II interactors such as the S1P1 specific compound CYM-5442 are allosteric regulators whose interaction with the S1P1 receptor occurs in a binding pocket independent of the 'key' Arg-120 and Glu-121 residues necessary for S1P polar head-group interactions (Gonzalez-Cabrera et al., 2008; Schurer et al., 2008). It has been theorized that persistent signalling may be a result of changes in accessibility to the binding pocket following activation caused by small rearrangement of transmembrane helices. This may alter or prevent ligand egress from the binding pocket as seen with the opsin receptor (Hildebrand et al., 2009; Schurer et al., 2008).

3.1.2 The role of S1P1 receptors in T-cells.

Gilenya® (Fingolimod; FTY720) has recently received FDA approval as a first line therapy for RRMS. In a brief summary of phase III clinical trials, both 0.5 and 1.25 mg doses of FTY720-P reduced annual relapse rates, disability progression as well as MRI end points compared to placebo and compared with a 30μg, once-weekly, intramuscular injection of interferon-β-1a (Cohen et al., 2010). The lower dose of 0.5 mg was as effective as the higher dose while providing the best risk-benefit ratio (Cohen et al., 2010; Horga et al., 2010). Both doses of the drug led a 70% reduction in circulating lymphocytes (Kappos et al., 2010). This can be explained by the reversible, peripheral lymphopenia caused by FTY720-P induced functional antagonism of S1P1 receptors on the surface of T-cells (Goetzl and Graler, 2004). FTY720-P binds to and causes a persistent internalisation of S1P1 receptors on T-cells. The internalised receptor becomes degraded and down-regulated at the mRNA level (Matloubian et al., 2004; Pinschewer et al., 2000). This reduction or elimination of the S1P1

receptor from the cell surface leads to the inability of the immune cell to respond to the S1P gradient established between lymphoid tissues and the circulating blood. S1P levels are higher in body tissues and fluids than in lymphoid tissues, this gradient is carefully established and maintained by the various enzymes, lyases and phosphatases involved in sphingolipid metabolism (**Fig.** 1.4)(Hla et al., 2008). The S1P gradient acts as an egress signal for T-cell by its signalling through S1P1 receptors. This negative effect on lymphocyte egress from secondary lymphoid organs is presumed to be the main mechanism of action for FTY720-P.

3.1.3 The role of S1P1 receptors in astrocytes.

Studies have established that FTY720-P crosses the BBB with great ease due to the compound's lipophilic properties. It has been shown that FTY720-P enters the CNS and localises to the white matter with preferential distribution along the myelin sheath (Foster et al., 2007). All S1P receptors (with the exception of S1P4 receptors) are expressed in all CNS cells to varying degrees (Brinkmann, 2007; Brinkmann et al., 2002). Bearing in mind in that FTY720-P can accumulate in the CNS and that all CNS resident cells express a plethora of S1P receptors, it has become pertinent to question whether the mechanism of action of FTY720-P is solely due to its effect on T-cells or due to a central effect on resident cells of the CNS. Astrocytes have been shown to express mainly S1P1 and S1P3 receptors with little or no expression of S1P2 receptors (Van Doorn et al., 2010; Wu et al., 2008). Some studies have shown that S1P5 receptor expression can be elicited by exposure of astrocytes to various growth factors (Rao et al., 2004). Immunohistochemical studies of S1P1 receptor localisation in post-mortem human brain tissue found the receptor to be mainly localised to astrocytes in the grey matter (Nishimura et al., 2010). Decreased S1P1 expression in hypertropic reactive astrocytes but increased expression in astrocytes associated with fibrillary gliosis of old infarctions was also shown (Nishimura et al., 2010). S1P3 receptors have also been shown to be upregulated in reactive astrocytes in both chronic-inactive and actively demyelinating MS lesions (Fischer et al., 2011).

3.1.4 S1P receptor signalling in astrocytes.

Activation of S1P receptors on astrocytes results in the activation of various downstream signalling cascades. S1P exerts a mitogenic effect on astrocytes promoting proliferation in an ERK-dependent manner (Pebay et al., 2001; Sorensen et al., 2003). The ERK pathway is also involved in astrocyte migration, PTX-sensitive S1P1 receptor specific agonism has been shown to promote astrocyte migration through the ERK signalling pathway (Mullershausen et al., 2007). S1P has been shown to induce the release of trophic factors such as fibroblast growth factor-2 (FGF-2) and glial cell-derived

neurotrophic factor (GDNF) from astrocytes in a PTX-sensitive manner (Bassi et al., 2006; Yamagata et al., 2003). S1P treatment of astrocytes causes a 10-fold increase in inositol phosphate (IP) levels which is only partially sensitive to pertussis toxin (PTX). This indicates that S1P receptor evoked increases in $_{i}Ca^{2+}$ levels in astrocytes are mediated by both G_q (S1P3 receptor) and G_i (S1P1 receptor) coupled receptors. Although the activation of PLC and generation of IP_3 is generally associated with the actions of the α-subunit of G_q coupled receptors, studies have shown that high concentrations of the βγ subunits of G_i coupled receptors can also activate PLC (Birnbaumer, 1992). Astrocytes are extensively coupled via gap junctions; connexin43 is the major astrocytic component of gap junction channels (Dermietzel et al., 1991). Loss of gap junctional communication between astrocytes and increased cellular proliferation are markers of reactive astrogliosis (Eugenin et al., 2012). Through its actions on both S1P1 and S1P3 receptors S1P is a potent inhibitor of gap junctional communication between astrocytes (Rouach et al., 2006). Activation of G_i signalling pathways including a reduction in cAMP and activation of ERK phosphorylation in addition to $G_{12/13}$ mediated activation of the Rho/ROCK signalling cascade results in an increase in non-phosphorylated connexin43 which inhibits the formation of functional gap junction channels (Rouach et al., 2006).

3.1.5 S1P receptors control of myelination states.

The role of S1P receptors in preventing demyelination and promoting remyelination has been investigated as an additional mode of action for Gilenya®. Modulation of S1P1 receptors has been shown to inhibit lysolecithin (LPC) induced demyelination in cerebellar slice cultures (Sheridan and Dev, 2012). This effect correlated with an inhibition in the release of several pro-inflammatory chemokines from the slice cultures (Sheridan and Dev, 2012). A separate study has shown that agonism of S1P5 receptors by FTY720-P increases levels of myelin basic protein (MBP) in myelinated neurospheres treated with LPC (Jackson et al., 2011). However other studies have failed to show any FTY720-P induced effect on remyelination in both *in vivo* cuprizone and LPC models of demyelination (Hu et al., 2011; Kim et al., 2011). FTY720-P agonism of S1P receptors has been shown to enhance process extension and cell survival in human oligodendrocyte progenitor cells (OPC) (Miron et al., 2008a). Moreover FTY720-P has a direct protective effect on OPCs, preventing apoptotic cell death induced by conditioned media from activated microglia and growth factor deprivation (Coelho et al., 2007). This cell survival was associated with activation of both ERK1/2 and PI3K/Akt signalling pathways and this protective effect was blocked by inhibition of these pathways (Coelho et al., 2007). It is possible that S1P receptor mediated, direct anti-apoptotic protection of oligodendrocyte progenitor cell pools may eventually lead to the replacement of lost mature oligodendrocytes, thus

indirectly enhancing remyelination. It is thought that while FTY720-P may not have any inhibitory effect on the demyelination process but it can enhance remyelination by dampening the activation of microglia and inhibiting the release of pro-inflammatory cytokines and nitric oxide from astrocytes (Jackson et al., 2011).

3.1.6 Role of astrocytic S1P1 receptors in vivo.

Astrocyte accumulation in the injured spinal cord can lead to formation of a glial scar which impedes repair and recovery processes. In an *in vivo* model of spinal cord injury FTY720-P treatment was shown to reduce vascular permeability and astrocyte accumulation (Norimatsu et al., 2012). *In vivo* studies of analgesic tolerance and hyperalgesia have shown that S1P generated from spinal astrocytes and microglia enhances the production of pro-inflammatory cytokines and nitric oxide from the surrounding glia (Muscoli et al., 2010). Inhibition of SphK1 and 2 enzymes ameliorated this increase in glial derived spinal cytokine formation (Muscoli et al., 2010). Genetic deletion of S1P1 receptors from various CNS cell types revealed astrocytic S1P1 to play a significant role in an EAE setting (Choi et al., 2011). Loss of S1P1 signalling by S1P1 deletion was shown to ameliorate EAE. This clinical improvement correlated with reduced pro-inflammatory cytokine expression, astrogliosis, demyelination, and axonal damage (Choi et al., 2011). This study gives a clear indication that modulation of astrocytic S1P1 receptors contributes to the clinical efficacy of FTY720-P, however it is worth noting that in an MS setting S1P1 receptors are not "deleted" but merely re-located. A number of caveats exist for this "functional antagonism" theory. For instance it does not take into account the persistent signalling of internalised or in fact, membrane associated S1P1 receptors (Mullershausen et al., 2009). There exists a pronounced disconnection between levels of lymphopenia and clinical scores in animal models whereby clinical improvement has been observed before the establishment of significant lymphopenia (Webb et al., 2004). This data would indicate the existence of a secondary clinically relevant, non-immunological target for FTY720-P. We believe that CNS expressed S1P receptors represent a viable pharmacological target for FTY720-P. The aim of this study is to examine the effect of FTY720-P on the internalisation and trafficking of endogenously expressed S1P1 receptors on primary rat astrocytes.

3.1.7 S1P receptor internalisation.

The intrinsic receptor properties including its innate ability to interact with either kinases or other regulatory proteins will govern how that receptor behaves following endocytosis. Small changes in receptors be it genetic, post-translatory or conformational can alter receptor affinity for β-arrestin,

signalling properties and post-endocytotic fate (Shiina et al., 2001). There exists at least three separate means of GPCR internalisation following agonist activation, all leading to the rapid removal of the GPCR from the cell membrane. Two main intracellular internalisation machineries are generally involved; these are (a) clathrin-coated pits and (b) the caveolae system. It has been suggested that differential sites of phosphorylation can lead to selection of different internalisation pathways (Rapacciuolo et al., 2003). The exact internalisation process of the S1P1 receptor has not yet been agreed on. While some studies suggest a role for the caveolae system, internalisation through clathrin coated pits would appear to be the more likely path of internalisation (Means et al., 2008; Watterson et al., 2002). Ubiquitination represents an important molecular event mediating arrestin and dynamin-dependent receptor endocytosis. Ubiquitination of both the arrestin protein and the GPCR is crucial to the internalisation and degradation of the receptor (Marchese and Benovic, 2001; Shenoy et al., 2001). Studies have shown differential levels of S1P1 receptor ubiquitination when bound by S1P and FTY720-P, with agonism of the receptor by FTY720-P leading to poly-ubiquitinated state versus mono-ubiquitination following activation by S1P (Oo et al., 2007). Previous truncation and mutation experiments have implicated the 351-SRSKSDNSS-359 serine-rich region (SRR) (**Fig.** 3.1) as the principal sequence governing agonist-induced internalisation, recycling and degradation of the S1P1 receptor (Oo et al., 2007; Watterson et al., 2002) Studies indicate a role for G-protein receptor kinase 2 (GRK2) and PKC mediated phosphorylation of the SRR leading to β-arrestin binding, uncoupling of the G-proteins and eventual internalisation (Oo et al., 2007; Watterson et al., 2002). Indeed a recent study has highlighted the importance of certain c-terminal serine and lysine residues in the post-translational modification of the S1P1 receptor (Oo et al., 2011). By mutating serine residues to non-phosphorylatable alanine residues 6 serine residues, 5 of which are in the previously described SRR (**Fig.** 3.1) were identified as necessary for FTY720-P induced degradation (Oo et al., 2011). In addition to this the group mutated several key lysine residues within the c-terminus to non-ubiquitinatable arginine residues (Oo et al., 2011). The data shows that when all four lysine residues are mutated the S1P1 receptor is resistant to FTY720-P induced degradation. Phosphorylation of these serine residues is necessary for subsequent FTY720-P induced polyubiquitination of the S1P1 receptor (Oo et al., 2011).

3.1.8 Role of the c-terminus in S1P1 receptor internalisation.

The extreme c-terminus of receptors has long been implicated in interacting with the molecular machinery necessary for receptor internalisation, these interactions are important for targeting receptors to either a recycling or degradative pathway. The protease-activated receptor 1 (PAR1) is a

GPCR that is targeted to lysosomes upon internalisation. Studies on the PAR1 receptor have identified the extreme c-terminal tail as the region that controls receptor sorting (Trejo and Coughlin, 1999). The c-terminal tail of the PAR1 receptor interacts with sorting nexin 1 which is involved in lysosomal targeting. Disruption of this interaction prevents agonist induced receptor degradation (Wang et al., 2002). Another important mechanism that governs receptor trafficking is that based on PDZ-dependent interactions. PDZ (PSD-95, Dlg, ZO-1 homology)-binding domain containing proteins can interact with c-terminally located PDZ motifs of various known receptors to alter receptor localisation, surface expression and receptor phosphorylation (Dev, 2007). For example, through its interaction with the c-terminus of glutamate receptors including the GluR-2 subunit of the AMPA receptor (Dev et al., 1999), GluR-5 and 6 subunits of the kainate receptor (Cho et al., 2003) and the metabotropic glutamate receptor mGluR-7 (Dev et al., 2000) the PDZ domain containing protein PICK1 can alter the surface expression and phosphorylation state of these receptors. In another example, phosphorylation of a serine residue in the c-terminally located PDZ motif of the β_2-adrenergic receptor by GRK5 disrupts the receptor's interaction with the PDZ-binding domain protein ezrin-radixin-moesin (EBP50) which results in the receptor being targeted to a degradative pathway (Cao et al., 1999). We hypothesise that interference in the interaction between unknown interacting proteins and the extreme c-terminus of the S1P1 receptor could alter FTY720-P induced internalisation and receptor fate. The aim of this study is to exam the role of the extreme c-terminus 372-MSSGNVNSSS-382 of the S1P1 receptor in FTY720-P induced internalisation in primary rat astrocytes.

3.1.9 Chapter Aim.

In the concluding remarks of a previous publication by Dev et al the question was posed "Does FTY720-P cause S1P receptor internalisation on brain cells similar to T-cells?" Answering this particular question became one of the main aims of this study. It has been shown that stably expressed S1P1 receptor in Chinese hamster ovary cells and endogenously expressed S1P1 receptor in human umbilical vein endothelial cells become internalised to a peri-nuclear region when treated with FTY720-P (Mullershausen et al., 2009). The first aim of this study was to investigate the effect of FTY720-P and other S1P receptor modulators on S1P1 receptor localisation and trafficking in primary rat astrocytes. A small TAT-based blocking peptide (MNP301) was also designed to competitively block possible protein-protein interactions at the extreme c-terminus of the S1P1 receptor. This blocking peptide was then utilised as a tool to investigate the importance of the c-terminus of the S1P1 receptor in receptor internalisation and trafficking in astrocytes.

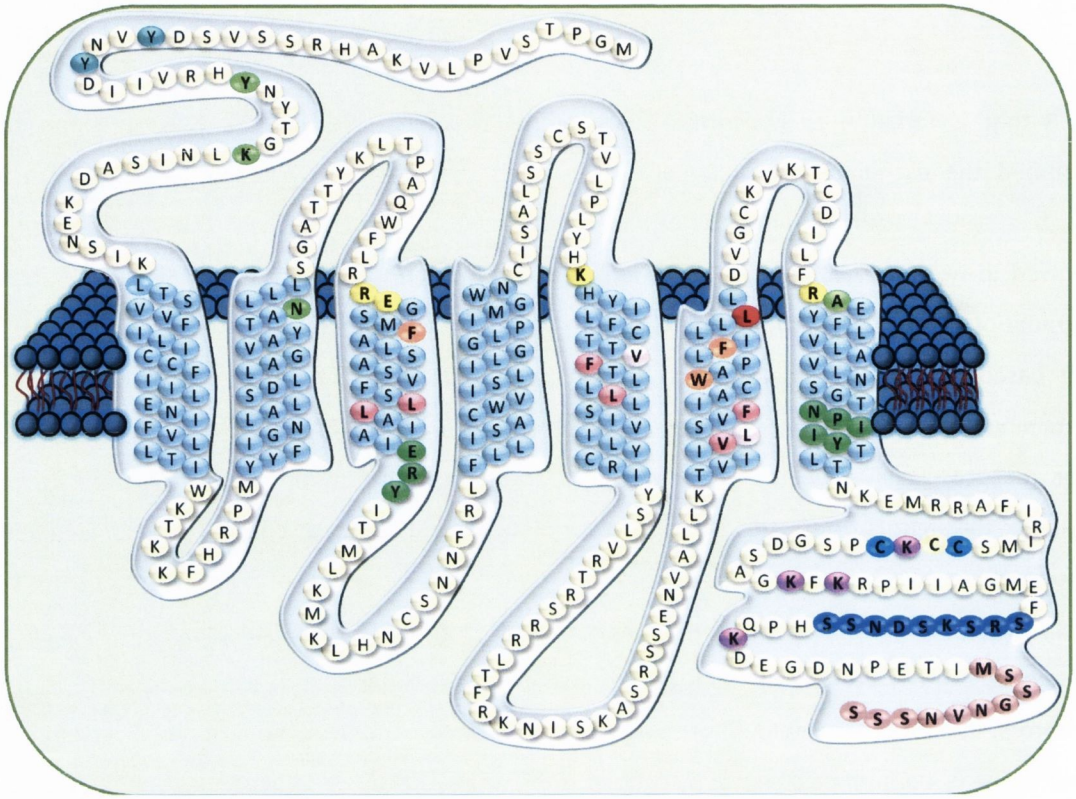


Figure 3.1 *Full amino acid sequence of Homo sapiens (human) S1P1 receptor.*

D(E)RY in helix 3 and NPXXY in helix VII are highly conserved domains that are essential for the transformation of the receptor from an inactive to a G-protein coupled conformation. S-palmitoylation sites on cysteine residues. Serine rich region (SRR), motif that has been shown to be essential for FTY720-P mediated receptor internalisation and degradation. Extreme c-terminus, also rich in phosphorylation sites. Ubiquitinylated lysine residues. Residues that make van der Waals contacts with each other and the alkyl chain of S1P. Hydrophobicity at V-209 and L-262 is necessary for the conformational change to the fully activated state. S1P1 receptor's L-276 is found in the hydrophobic binding pocket. Arg-120, Lys-200, and Arg-292 are found in the S1P1 and S1P4 receptors and form salt bridges with the S1Ps phosphate group, Glu-121 is required for S1P1 binding of S1P. Residues involved in the binding of sphingosine-like head groups. F-273, W-269 and F-125 residues are particularly important for receptor signalling, with W-269 being associated with ligand induced ERK phosphorylation.

3.2.2 FTY720-P does not alter GFAP or S1P1 receptor expression in astrocytes.

Before investigating the effects of FTY720-P on S1P1 receptor internalisation, the purity of primary rat astrocyte cultures was assessed using antibodies against markers of astrocytes (GFAP), neurons (neurofilament H), microglia (CD11b) and oligodendrocytes (CNPase) and the Hoechst nuclear stain to determine the total cell count (**Fig.** 3.2A). GFAP is a cellular marker for astrocytes and immunocytochemical presence of GFAP staining is indicative of the presence of astrocytes. Total cell counts were obtained using nuclear counts by way of Hoescht staining (blue). GFAP positive cells (green) were expressed as a percentage of total cell counts (**Fig.** 3.2B). Average percentage of positively stained cells for each group was as follows: GFAP 98.58% +/- 0.57, CD11b 1.35% **+/-** 0.77 and CNPase 1.83% **+/-** 0.48. No neurofilament H positive cells were observed. Cultures were found to be >98% pure confirming the high astrocytic purity of the culture (**Fig.** 3.2B). The expression of S1P1 receptors was also confirmed in astrocytes as determined by co-localisation of S1P1 receptor and GFAP immunoreactivity (**Fig.** 3.2C). The effect of FTY720-P on astrocyte viability and overall S1P1 receptor number was assessed by Western blotting. Cells treated with vehicle or with 1μM FTY720-P for 1hr were subjected to SDS-PAGE and Western blotting using S1P1 receptor and GFAP antibodies. Furthermore the data showed that FTY720-P did not alter GFAP (Top panel, **Fig.** 3.2D) or S1P1 receptor (Lower panel, **Fig.** 3.2D) expression levels in astrocytes.

3.2.3 FTY720-P treatment causes S1P1 receptor internalisation in pure astrocytic cultures in a concentration dependent manner.

Initial experiments were conducted on pure astrocyte cultures to determine the effect of the FTY720-P on S1P1 receptors expressed on these glial cells. Astrocytes were treated with increasing concentrations of FTY720-P (10nm, 100nm, and 1μM) for 1hr at 37°C to assess the effect of FTY720-P on the internalisation state of S1P1 receptors. Following compound treatment, these cells were subjected to a standard immunocytochemical protocol, probing for the S1P1 receptor (green) and GFAP (red). FTY720-P treatment caused internalisation of the S1P1 receptor to a peri-nuclear compartment as confirmed by confocal microscopy (**Fig.** 3.3). It was found that increasing concentrations of the drug caused increasing levels of internalisation, treatment with 1μM concentration resulted in maximal internalisation. This concentration was utilised hence forth as the working concentration.

3.2.4 FTY720-P treatment of pure astrocyte cultures causes S1P1 receptor internalisation in a time dependent manner.

Following experiments testing the effect of varying concentrations of FTY720-P on S1P1 receptor internalisation, the kinetics of FTY720-P induced S1P1 receptor internalisation were assessed. The effects of 1μM FTY720-P were measured at different time points. Pure astrocyte cultures were treated with 1μM FTY720-P at 37°C for (5mins, 10mins, 30mins, 1hr and 4hr). Increasing drug treatment times caused increasing levels of S1P1 receptor internalisation (**Fig.** 3.4). Effective internalisation was clearly evident after 1hr drug treatment (**Fig.** 3.4E) and remained after 4hr treatment. Overall fluorescence appeared to be reduced with a 4hr treatment indicating possible receptor degradation (**Fig.** 3.4F).

3.2.5 FTY720-P treatment of pure astrocyte cultures causes persistent internalisation of the S1P1 receptor.

Following previous observations that FTY720-P causes rapid and persistent internalisation of the S1P1 receptor subtype in Chinese hamster ovary (CHO) cells stably expressing S1P1 receptors and in primary human umbilical vein endothelial cells (HUVECs) expressing endogenous S1P1 receptor, this event was investigated in primary rat astrocyte cells. Astrocytes treated with FTY720-P (1μM) for 1hr at 37°C caused S1P1 receptor internalisation to a peri-nuclear region as described above (**Fig.** 3.5A). Moreover, under conditions of FTY720-P treatment (1μM) for 1hr at 37°C, followed by a 5hr washout period, the S1P1 receptor remained localised in intracellular sites (**Fig.** 3.5A), and in some cases more prominently than after 1hr FTY720-P treatment. In agreement, automated image analysis showed a significant increase in the ratio of internalisation between the vehicle control and both the 1hr FTY720-P treatment group (* $p<0.05$, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 3.5C) and the 5hr washout group (*** $p<0.001$, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 3.5C). Taken together, the data showed that FTY720-P induced a persistent state of internalisation of S1P1 receptor to a perinuclear region in astrocytes, similar to CHO and HUVECs.

3.2.6 Internalised S1P1 receptor colocalises with the Golgi matrix and trans-Golgi-network.

The data showed that the S1P1 receptors are internalised to a peri-nuclear region following exposure to FTY720-P. To examine the intracellular localisation of internalised S1P1 receptors, astrocytes treated with FTY720-P (1μM) for 1hr at 37°C were stained with various organelle markers. Staining with the early endosome marker (EEA-1) showed no colocalisation with the internalised S1P1 receptor (**Fig.** 3.6). In addition, counter staining with the lysosome marker (LAMP1) also

showed minimal colocalisation with the intracellular pool of S1P1 receptors (**Fig.** 3.6). In contrast, the data showed considerable overlap of S1P1 receptor staining with both markers for the trans-Golgi-network (TGN) (P230) and the Golgi complex (GM130) (**Fig.** 3.6). The results indicated that FTY720-P induced internalised S1P1 receptor is trafficked to the TGN or Golgi matrix and are in agreement with previous studies showing the localisation of FTY720-P induced internalised S1P1 receptors in HUVECs (Mullershausen et al., 2009).

3.2.7 S1P1 receptor agonists FTY720-P and SEW2781 cause S1P1 receptor internalisation.

To further investigate internalisation of the S1P1 receptor, primary astrocyte cultures were treated with three S1P1 receptor agonists; S1P, the endogenous ligand, FTY720-P, an S1P mimetic and non-selective S1P1 agonist and SEW2871, a synthetic S1P1-selective agonist. Cells were starved in serum-free media for 3hr prior to treatment to prevent any serum derived S1P from interfering with the experiment. Cells were treated for 1hr with 1μM compound concentration at 37°C, dimethyl sulfoxide (DMSO) was used as a vehicle control. Distribution of the S1P1 receptor was assessed using immunocytochemistry and confocal microscopy. High through-put image analysis showed that both FTY720-P and SEW2871 caused significant internalisation of the S1P1 receptor compared to control (*** $p<0.001$, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 3.7B). S1P did not cause internalisation after a 1hr treatment. S1P stimulated S1P1 receptor may have been internalised into endosomal compartments, stripped of its ligand and recycled back to the plasma membrane prior to fixation. Collectively the data suggested that FTY720-P differentially modulates S1P1 receptors compared to the endogenous ligand S1P in astrocytes.

3.2.8 MNP301 transduces astrocytes in a concentration-dependent manner.

In order to uncouple the agonist effects of FTY720-P from its effects on S1P1 receptor internalisation, we set out to develop a compound that prevents FTY720-P mediated S1P1 receptor internalisation. We hypothesised that the c-terminus of the S1P1 receptor interacts with trafficking proteins that regulate receptor cycling. Here, we report the development of a biologically active peptide (MNP301). Specifically, MNP301 is modelled on the last 10 residues of c-terminus of the S1P1 receptor, fused to a protein transduction domain (PTD) based on the HIV trans-activating transcriptional activator (TAT) sequence for cell delivery and labelled with FITC for visualisation inside the cell (FITC-YGRKKRRQRRR-MSSGNVNSSS) (**Fig.** 3.8A). Primary astrocytes cultures were treated with increasing concentrations of MNP301 (0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml) for 1hr at 37°C. Cells were washed prior to fixation to remove any extracellular attached peptide.

Visualisation of the peptide was facilitated by the FITC fluorescent tag. Confocal microscopy and automated image analysis showed that MNP301 transduces astrocytes in a concentration dependent manner (**Fig.** 3.8B). Effective transduction was observed following incubation with 100μg/ml of MNP301. This 100μg/ml group showed significant cellular transduction compared to control (*** $p<0.001$ versus 0.1μg/ml and 1μg/ml, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 3.8C). This result confirms the intracellular availability of MNP301 and allows for the observation of any peptide associated effects on S1P1 receptor internalisation and trafficking.

3.2.9 MNP301 transduces astrocytes in rat organotypic cerebellar slice cultures.

To further investigate the ability of MNP301 to transduce cells, rat organotypic cerebellar slice cultures were incubated with MNP301 (250μg/ml) for 2hr. Peptide was then removed and slices left in fresh media for a 2 days. Following this slices were fixed and stained for GFAP (grey) and the S1P1 receptor (purple). Data further confirms the cellular transduction ability of MNP301. Peptide was found to remain within cells following a two day wash out period, as seen by FITC fluorescence (green) (**Fig.** 3.9).

3.2.10 MNP301 alters FTY720-P induced internalisation of the S1P1 receptor.

Following validation of MNP301's transduction of astrocytes, MNP301's effect on FTY720-P induced S1P1 receptor internalisation was investigated. Primary rat astrocytes were subjected to a 4hr starvation period in serum free medium, cells were then pre-incubated with 100μg/ml MNP301 for 2hr. The media was then removed and cells washed x3 with serum free media to eliminate excess peptide. A final concentration of 1μM FTY720-P was added to the astrocytes for 1hr at 37°C/5% CO2, following this cells were washed, fixed, permeabilised, blocked and stained for GFAP and S1P1 receptor. It was found by way of immunocytochemistry and confocal microscopy that pre-incubation of astrocytes with MNP301 attenuated internalisation of the S1P1 receptor normally induced by FTY720-P (**Fig.** 3.10A). Immunocytochemistry coupled with high through-put image analysis confirmed qualitative assessment showing pre-incubation with the peptide significantly attenuated FTY720-P induced receptor internalisation to the peri-nuclear region (*** $p<0.001$ vs. FTY720-P treatment alone, one-way ANOVA, Bonferroni post-hoc test) (**Fig.** 3.10B). This data suggests that possible protein interactions at the extreme c-terminus of the S1P1 receptor are necessary for internalisation of the receptor. The data also shows that a peptide modelled on the last 10 amino acids of the S1P1 receptor can prevent FTY720-P induced S1P1 internalisation to the TGN. Also it was noted that overall fluorescence was decreased in astrocytes that were treated with both MNP301

and FTY720-P, indicating possible receptor degradation. It is possible that MNP301 alters the cellular fate of FTY720-P bound S1P1 receptors causing the receptors to be degraded instead of accumulating in intracellular compartments.

3.2.11 An unrelated peptide MNP201 has no effect on FTY720-P induced internalisation of the S1P1 receptor.

To test the specificity of MNP301's ability to alter the internalisation of FTY720-P bound S1P1 receptors, primary rat astrocytes were treated with a TAT-based FITC tagged unrelated peptide, MNP201 (FITC-YGRKKRRQRRRVCMGDHWFDV). As before a 100μg/ml concentration of MNP301 prevented the trafficking of internalised S1P1 receptors to the TGN (**Fig.** 3.11A). In contrast a pre-treatment of rat astrocytes with a 100μg/ml concentration of MNP201 had no effect on FTY720-P bound S1P1 receptors as shown by confocal microscopy (**Fig.** 3.11B). The data indicates that the effect of MNP301 on S1P1 receptor internalisation is specific. A peptide of the same size (21 residues) as MNP301 that is both TAT linked and FITC-tagged had no effect on FTY720-P induced S1P1 internalisation thereby excluding possible influence of the TAT epitope or FITC tag.

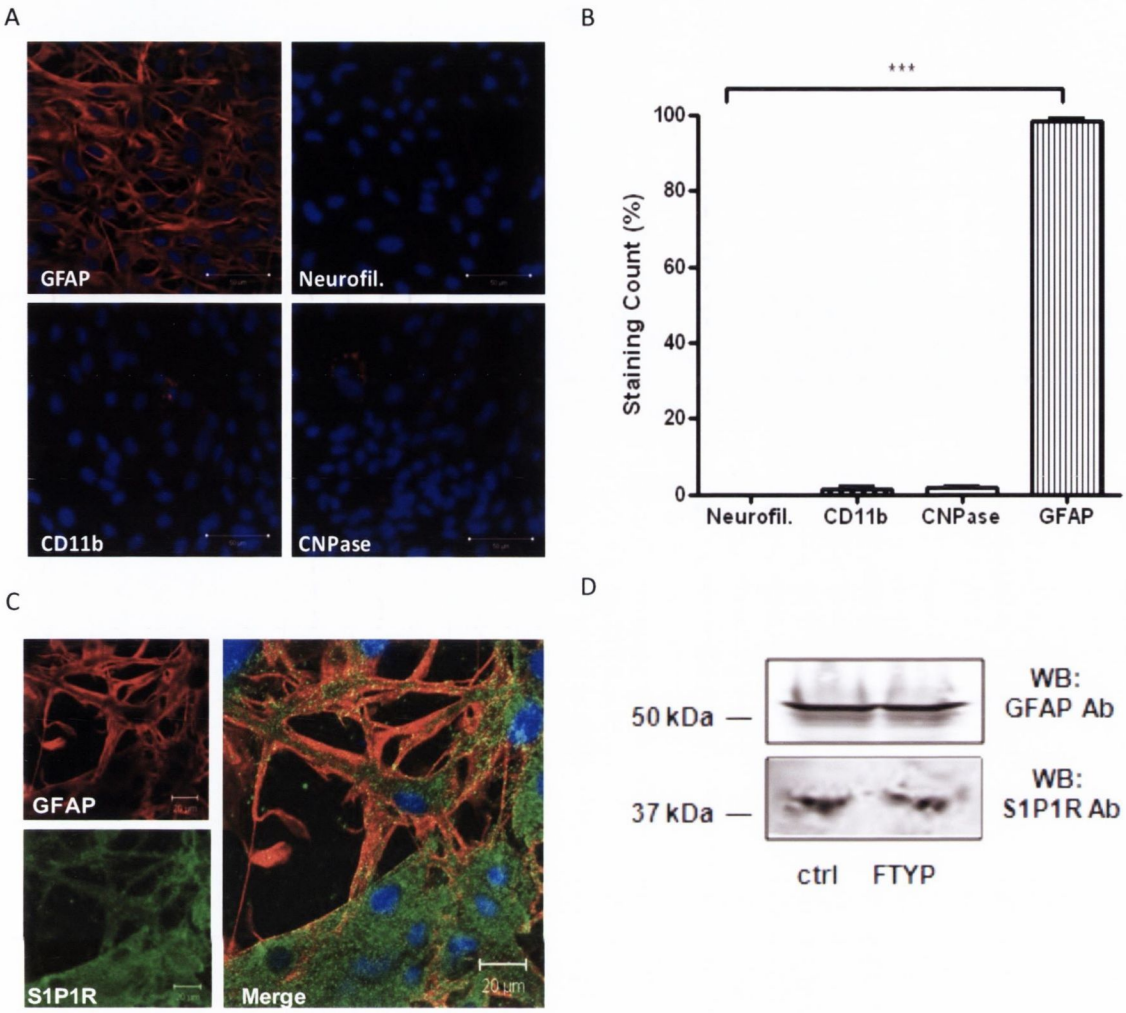


Figure 3.2 *FTY720-P does not alter GFAP or S1P1 receptor protein levels.*

(A) Pure astrocyte preparation stained for GFAP (astrocytes), neurofilament H (neurons), CD11b (microglia) and CNPase (oligodendrocytes). Cell nuclei appear as blue (Hoescht). A total of 24 images were analysed (6 images per group). **(B)** Average percentage of positively stained cells for each group was as follows: GFAP 98.58% +/- 0.57, CD11b 1.35% **+/-** 0.77 and CNPase 1.83% **+/-** 0.48. No neurofilament H positive cells were observed. **(C)** Pure astrocyte preparation stained for GFAP and S1P1 receptor, showing expression of S1P1 receptors in astrocytes. Scale bars 50 µm. **(D)** Western blots depict similar GFAP (51 kDa) and S1P1 receptor (38 kDa) expression levels, pre- and post-FTY720-P (1µM) treatment. Scale bars 50µm.

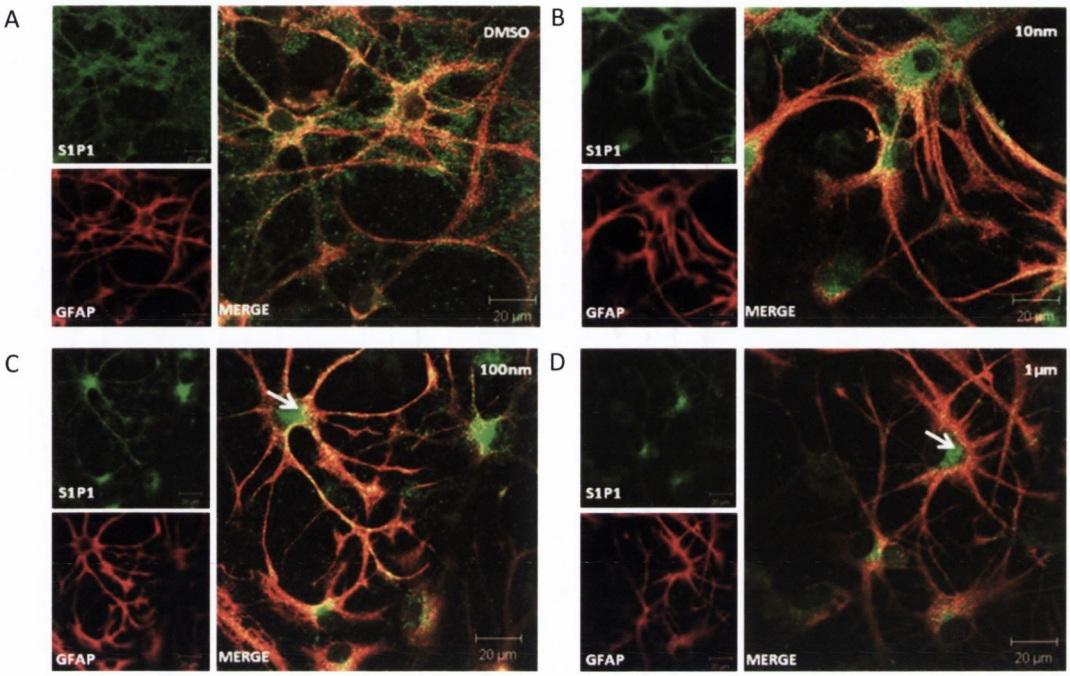


Figure 3.3 *FTY720-P treatment causes S1P1 receptor internalisation in pure astrocytic cultures in a concentration dependent manner.*

Astrocytic cultures were treated with increasing concentrations of FTY720-P. **(A)** DMSO, **(B)** 10nM FTY720-P, **(C)** 100nM FTY720-P, **(D)** 1µM FTY720-P. Arrows indicate areas of internalisation. Cells are immunostained with GFAP Ab (red) and S1P1 receptor Ab (green). Scale bars 20µm.

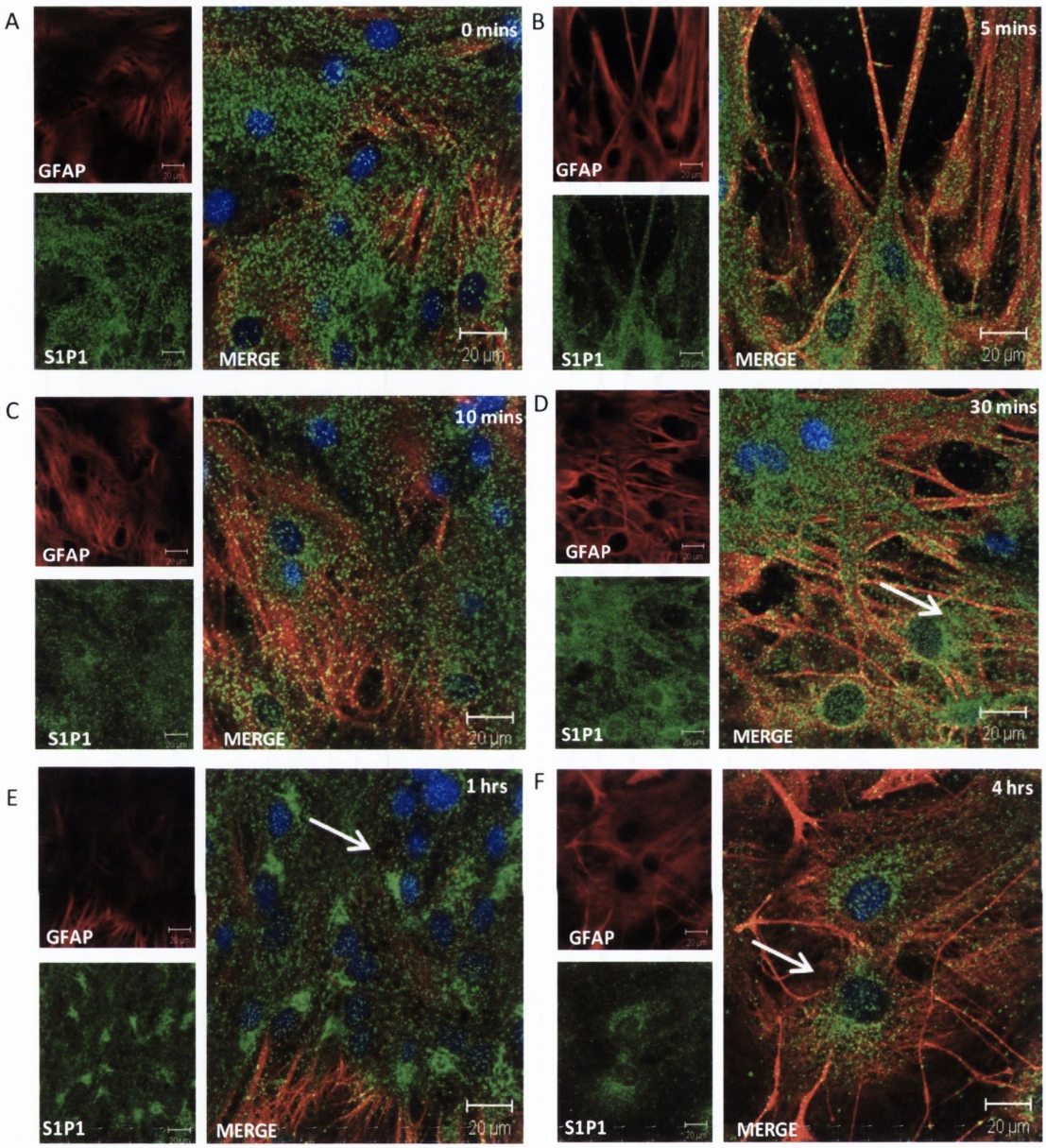


Figure 3.4 *FTY720-P treatment causes S1P1 receptor internalisation in pure astrocytic cultures in a time dependent manner.*

Pure astrocyte cultures were treated with 1µM FTY720-P. The drug was washed off after **(A)** 0min, **(B)** 5min, **(C)** 10min, **(D)** 30min, **(E)** 1hr and **(F)** 4hr. Arrows indicate areas of internalisation. Cells were immunostained with GFAP antibody (red) and S1P1 receptor antibody (green). Cell nuclei appear as blue (Hoescht). Scale bars 20µm.

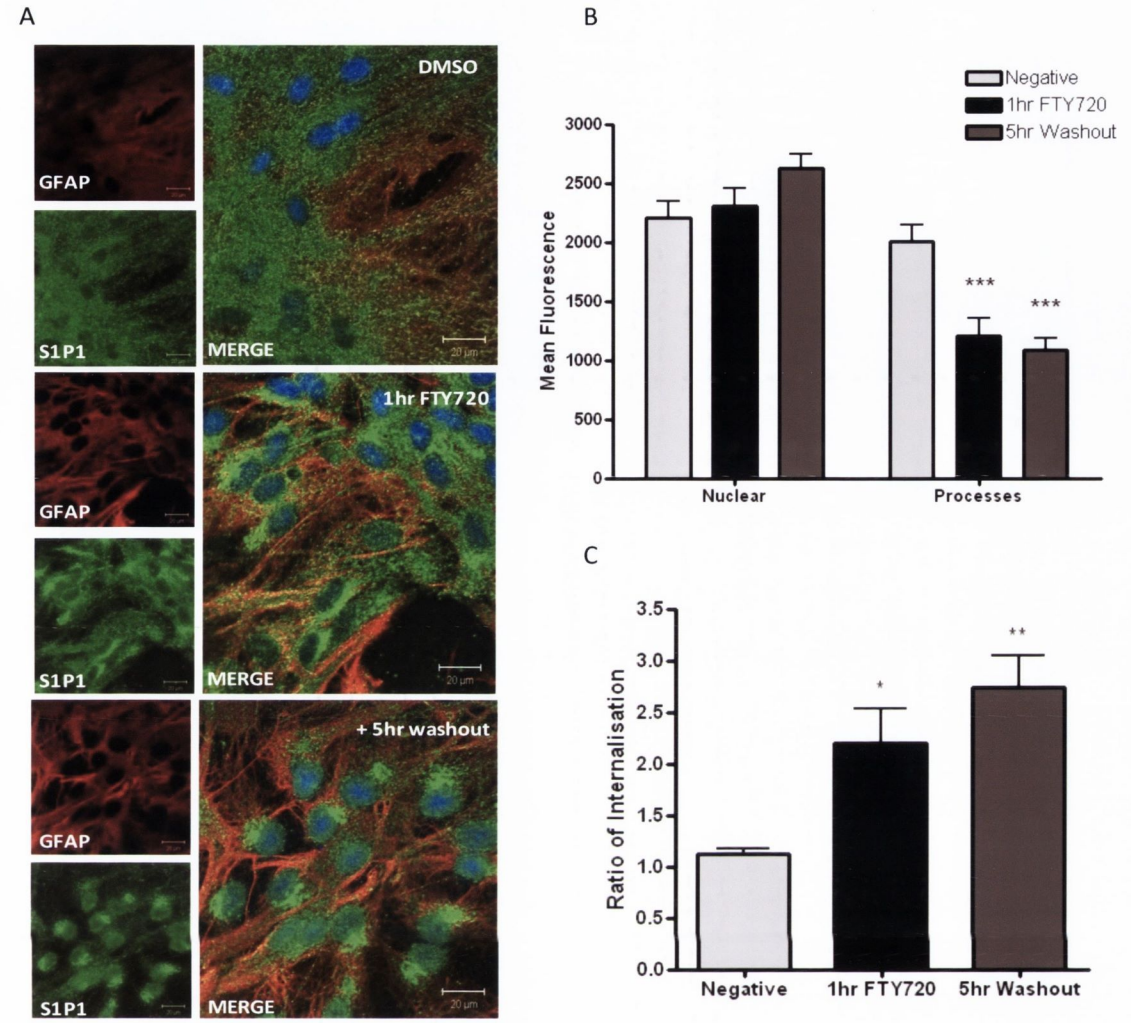


Figure 3.5 *FTY720-P treatment causes persistent internalisation of the S1P1 receptor.*

(A) Vehicle control (0.1% DMSO), 1μM FTY720-P treatment for 1hr (positive control) and 1μM FTY720-P treatment for 1hr followed by a 5hr washout period. Cells are immunostained with GFAP Ab (red) and S1P1 receptor Ab (green). Cell nuclei appear as blue (Hoescht). **(B)** and **(C)** Bar graph shows changes in the levels of nuclear and cytoplasmic S1P1 receptor staining in astrocytes treated with FTY720-P. Statistical analysis showed the level of internalisation, data expressed as mean +/- SEM (*** $p<0.001$, vehicle control vs. treatment, two-way ANOVA and Bonferroni post-hoc test). The ratio of nuclear:cytoplasmic S1P1 receptor expression was also calculated. (* $p<0.05$, ** $p<0.01$ vehicle control vs. treatment, one-way ANOVA and Dunnetts post-hoc test). The figure is representative of two independent experiments. Scale bars 20μm.

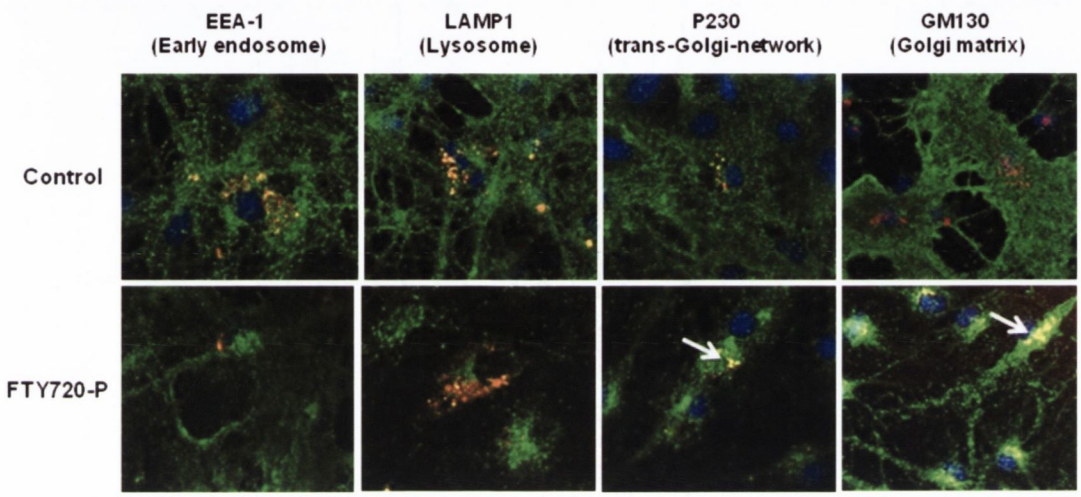
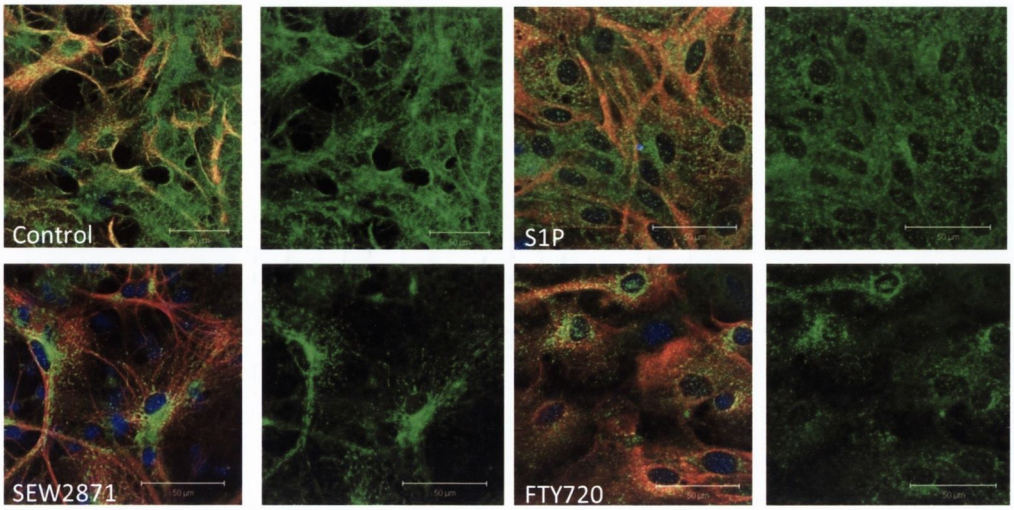


Figure 3.6 *FTY720-P treatment internalises S1P1 receptors to the trans-Golgi-network in rat astrocytes.*

Vehicle control and FTY720-P treated astrocytes were stained with S1P1 receptor antibody (green) and various organelle marker antibodies (red) including early endosome (EEA-1), trans-Golgi-network (P230), lysosome (LAMP1) and Golgi matrix (GM130). Cell nuclei appear as blue (Hoescht). Arrows indicate overlap of internalised S1P1 receptor with P230 and GM130 markers.

A



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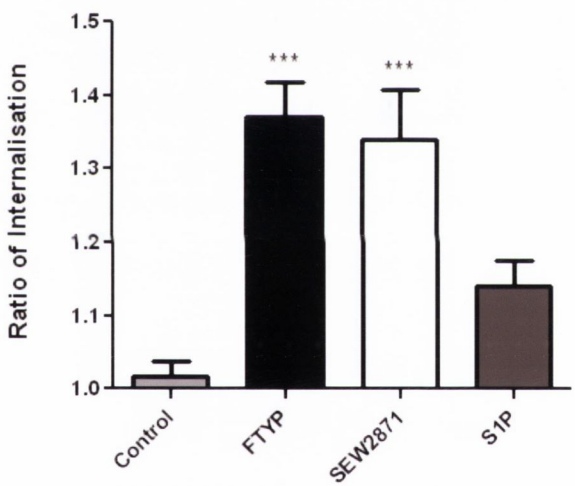


Figure 3.7 *The endogenous ligand S1P does not internalise S1P1 receptors.*

The distribution of the S1P1 receptor (green) was investigated following treatment with **(A)** DMSO (vehicle control), FTY720-P (1μM, 1hr), the selective S1P1 agonist SEW2871 (1μM, 1hr), and the endogenous ligand S1P (1μM, 1hr). Arrows indicate areas of internalisation. Cells are immunostained with GFAP antibody (red) and S1P1 receptor antibody (green). Cell nuclei appear as blue (Hoescht). **(B)** Statistical analysis showing level of internalisation (*** $p<0.001$, vehicle control vs. treatment, one-way ANOVA, Dunnetts post-hoc test). The figures are representative of two independent experiments. Scale bars 50μm.

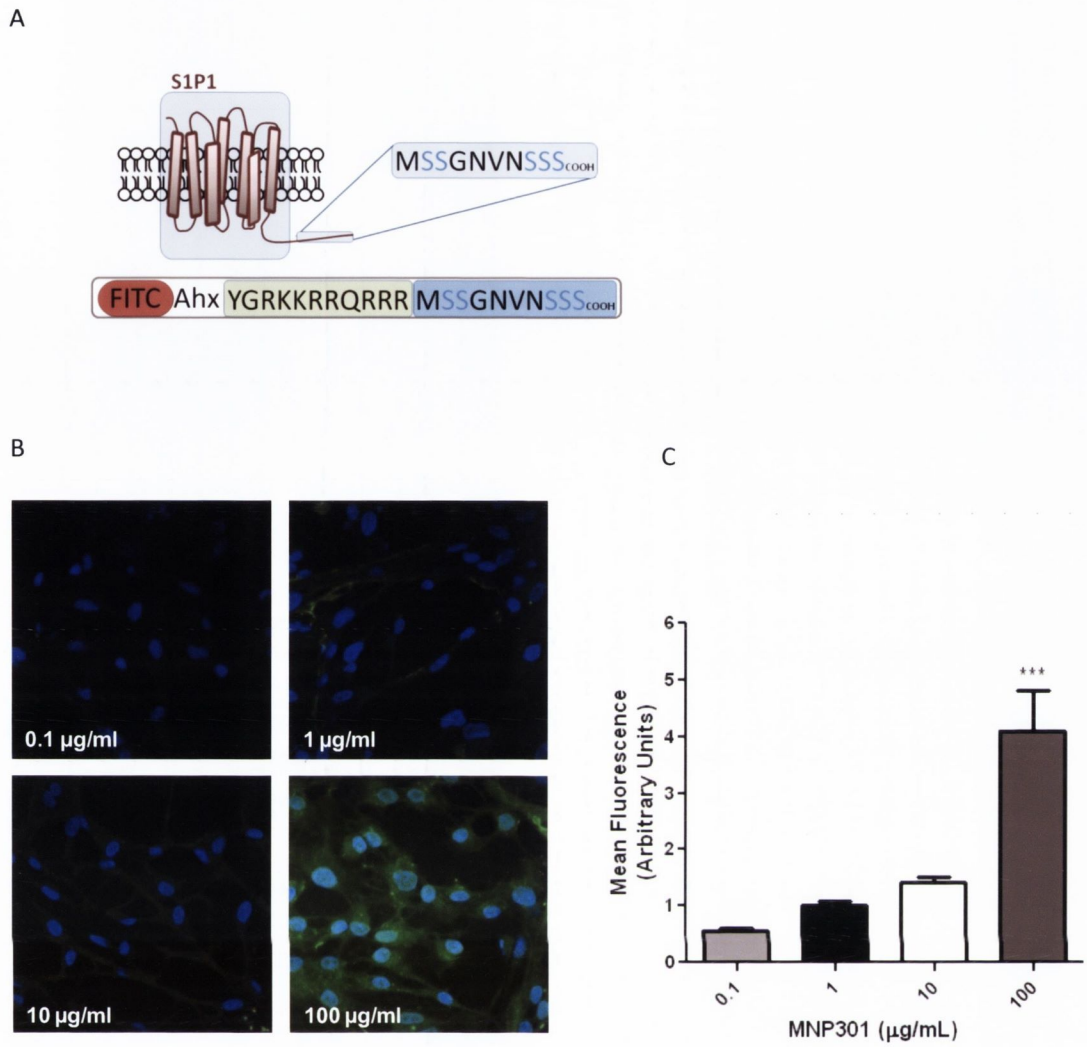


Figure 3.8 *MNP301 transduces primary rat astrocytes in a concentration dependent manner.*

The structure of MNP301 is shown **(A)** and is composed of a FITC tag, a cell transduction TAT sequence and the last 10 residues of c-terminus of the S1P1 receptor (FITC-Ahx-YGRKKRRQRRR-MSSGNVNSSS). **(B)** Pure astrocytes cultures were treated with MNP301 (0.1µg/ml, 1µg/ml, 10µg/ml and 100µg/ml) for 1hr at 37°C. Cells were washed, fixed and direct FITC fluorescence (green) was observed at a wavelength of 488 nm by confocal microscopy. Cell nuclei appear as blue (Hoescht). **(C)** Mean fluorescence was calculated from 6 images per condition. Significant cellular transduction was observed following incubation of astrocytes with 100µg/ml MNP301 compared to control (*** $p<0.001$ versus 0.1µg/ml, 1µg/ml and 10µg/ml, one-way ANOVA, Dunnetts post-hoc test). Experiment is representative of 2 separate experiments.

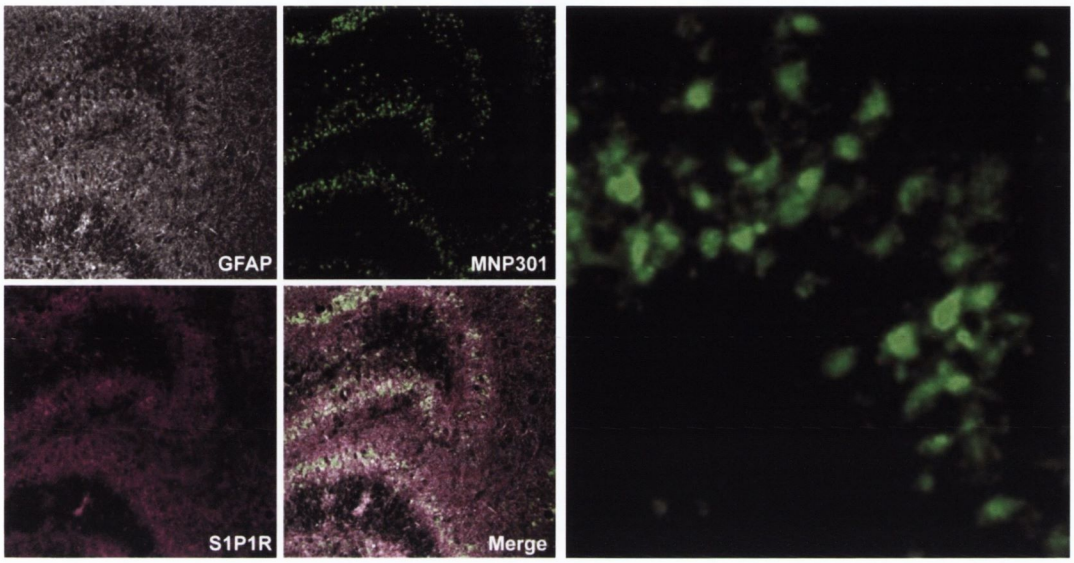


Figure 3.9 *MNP301 successfully transduces astrocytes in organotypic slice cultures.*

Rat organotypic cerebellar culture were treated acutely for 2hr with MNP301 (250μg/ml) and then placed into fresh medium for a further 2 days. The slices were then stained for S1P1 receptor (purple), GFAP (grey), MNP301 peptide was visible due to its FITC-tag (green). Qualitative data indicates transduction and cellular availability of MNP301 following incubation with organotypic cerebellar slice cultures. Note this is an observation from a single experiment.

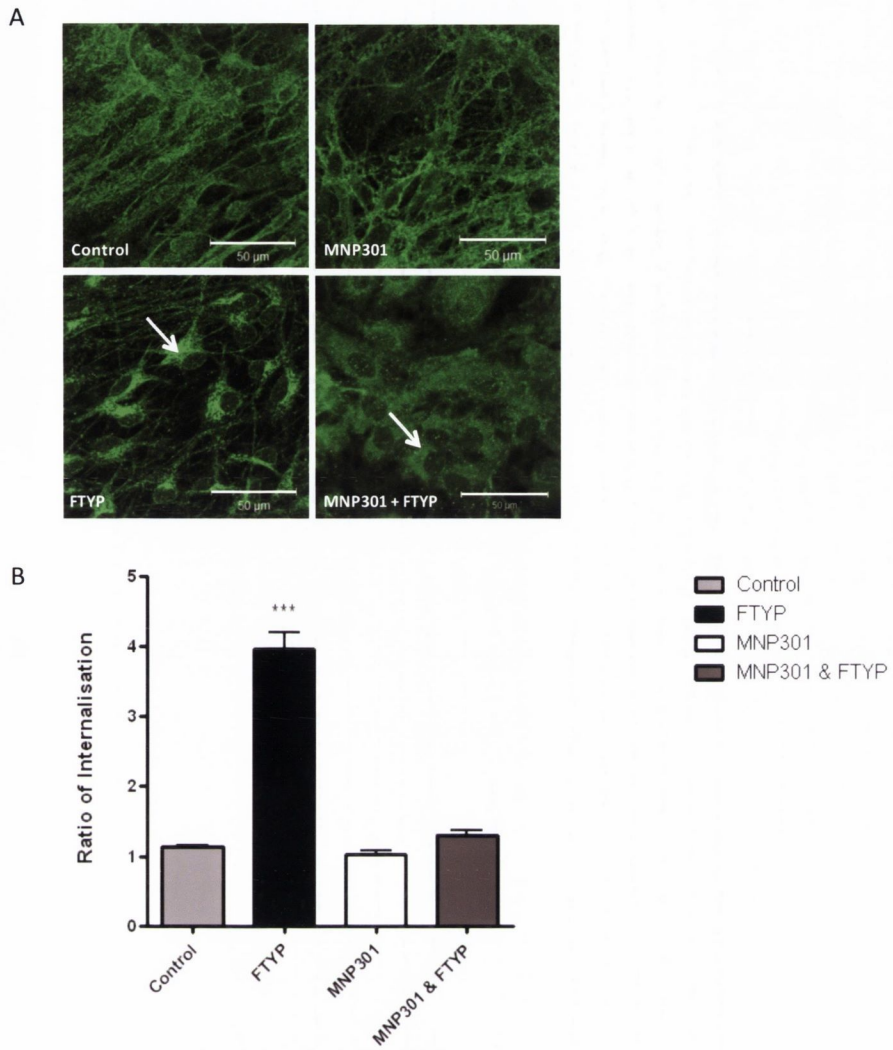
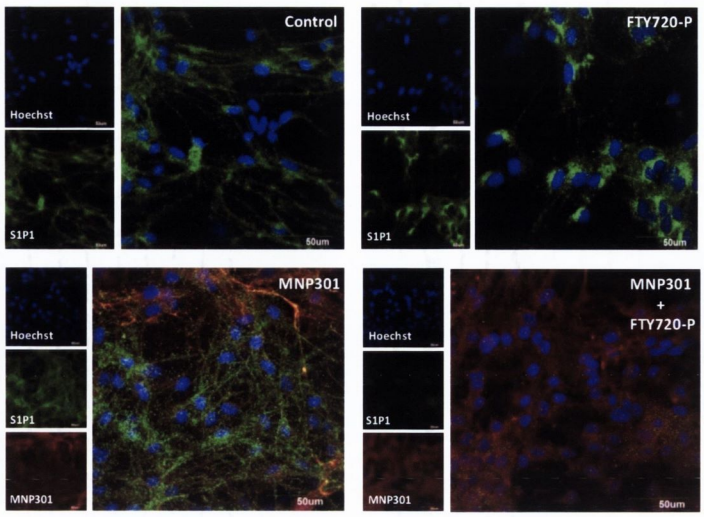


Figure 3.10 *MNP301 attenuates FTY720-P induced internalisation of S1P1 receptors in astrocytes.*

(A) Astrocytes were treated with FTY720-P (1µM) for 1hr in the presence and absence of 100µg/ml MNP301. Arrows indicate S1P1 receptor internalisation. Cells are immunostained for S1P1 receptor (green). **(B)** The analysed data shows that MNP301 treatment alone had no effect on S1P1 receptor internalisation, FTY720-P caused a significant increase in S1P1 receptor internalisation vs. control, and treatment of cells with FTY720-P following incubation with MNP301 attenuated the FTY720-P induced receptor internalisation. The number of cells analyzed per treatment group were; vehicle control = 154; MNP301 = 144; FTY720-P = 225; MNP301+FTY720-P = 264. (C) Statistical analysis of internalisation indicates that MNP301 significantly blocks FTY720-P induced internalisation to the TGN. Data expressed as mean +/- SEM, (*** $p<0.001$ vs. FTY720-P alone, one-way ANOVA, Bonferroni post-hoc test). Data is representative of 3 separate experiments. Scale bars 50µm.

A



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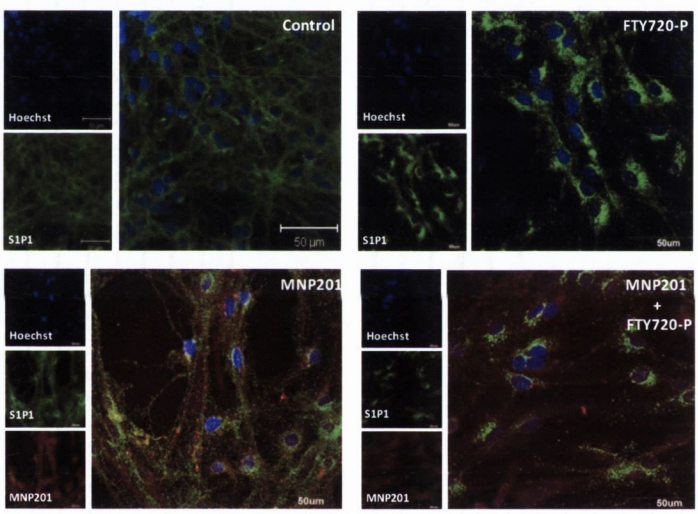


Figure 3.11 *MNP201 does not attenuate FTY720-P induced internalisation of S1P1 receptors.*

Astrocytes were treated with **(A)** FTY720-P (1µM) for 1hr at 37°C in the presence and absence of 100µg/ml MNP301. In addition, astrocytes were treated with **(B)** FTY720-P (1µM) for 1hr at 37°C in the presence and absence of 100µg/ml MNP201 (FITC-Ahx-YGRKKRRQRRR-VCMGDHWFDV). Cells are immunostained for S1P1 receptor (green) to examine receptor internalisation. MNP201 failed to alter the internalisation FTY720-P treated S1P1 receptors compared to the S1P1 specific peptide MNP301. Data is representative of 2 separate experiments. Scale bars 50µm.

3.3 Discussion

We have reported previously that modulation of S1P1 receptors by FTY720-P regulates intracellular signalling in rat astrocytes, including the inhibition of adenylyl cyclase, activation of phospholipase C, increase of ERK phosphorylation and rise of $_{i}Ca^{2+}$ levels (Mullershausen et al., 2007; Osinde et al., 2007). We have also demonstrated that S1P1 receptor activation promotes astrocyte migration (Mullershausen et al., 2007) in line with S1P-induced migration of neural stem cells and oligodendrocyte precursors cells (Kimura et al., 2007; Novgorodov et al., 2007). Evidence suggests a specific role for the S1P1 receptor subtype in astrocytes, as the effects of FTY720-P and S1P are mimicked by selective S1P1 receptor agonists and blocked by S1P1 receptor antagonists (Mullershausen et al., 2007; Osinde et al., 2007). The current study has contributed to our current knowledge of the interaction between S1P receptor agonists and the S1P1 receptor. We have shown that FTY720-P causes a time and concentration dependent persistent internalisation of S1P1 receptors in primary rat astrocytes. In addition we have demonstrated that these persistently internalised receptors localise to the TGN, this persistent internalisation can also be induced by the S1P1 specific agonist SEW2871, but not by the natural agonist S1P. Furthermore we have shown that agonist induced internalisation of the S1P1 receptor is susceptible to inhibition. We show that administration of a blocking peptide (MNP301) designed to match the last 10 amino acids of the extreme c-terminus of the S1P1 receptor can inhibit FTY720-P induced persistent internalisation of the S1P1 receptor. Finally this inhibition of receptor internalisation to the TGN was specific to MNP301 as a non related peptide of similar molecular properties did not have any effect on FTY720-P induced internalisation of the S1P1 receptor.

3.3.1 Does FTY720-P cause S1P receptor internalisation in brain cells similar to T-cells?

We show for the first time that FTY720-P can cause S1P1 receptor internalisation in a time and concentration dependent manner in primary rat astrocytes. By introducing a 5hr drug washout step we showed that a 1μM concentration of FTY720-P caused persistent internalisation of the S1P1 receptor which corroborates previous work done by groups using CHO cells (Mullershausen et al., 2009). This state of internalisation is not common with normal agonist stimulated GPCR's (**Fig.** 1.7), however it does mirror the 'functional antagonistic' effect achieved by FTY720-P on S1P1 receptors in T-cells. Whether any physiological effect of FTY720-P on astrocytes is due to direct agonism, as the receptor has been shown to continue to signal from intracellular compartments (Mullershausen et al., 2009), or due to functional antagonism remains unclear, and warrants investigation. Similar levels of internalisation were achieved when treating the cells with the S1P1 receptor agonist

SEW2871. Treatment of astrocytes with the S1P1 receptor's natural ligand S1P did not cause any noticeable internalisation. It is likely that S1P causes 'fast' recycling of the S1P1 receptor. Once internalised in the endosomal compartments, the receptor-agonist complex receives various sorting signals. FTY720-P induces poly- or exaggerated ubiquitinylation of the receptor which acts as signal to target the receptor to the proteasomal degradative pathway (Oo et al., 2007). This might be caused by a stronger binding affinity between FTY720-P and the S1P1 receptor compared to the S1P-S1P1 receptor complex. It is also possible that FTY720-P bound S1P1 receptor has a different conformational structure compared to the S1P-S1P1 receptor complex. Either of these events may influence the level of agonist bound receptor ubiquitinylation or phosphorylation leading to separate intracellular fates for the receptor.

3.3.2 Can this internalisation event be modulated by small peptide sequences?

We have also shown that FTY720-P induced persistent internalisation of the S1P1 receptor is prone to modulation. A small blocking peptide was utilised to further understand the relationship between FTY720-P and the S1P1 receptor. The blocking peptide (MNP301) is 22 amino acids in length with an 11 amino acid TAT sequence which permits uptake of the peptide by the cell. Fused to this TAT sequence is a 10 amino acid stretch that corresponds to the extreme c-terminus of the S1P1 receptor (**Fig.** 3.1). The data showed that administration of this peptide attenuates FTY720-P induced S1P1 receptor internalisation to the TGN. This 'blocking peptide effect' is specific to MNP301 as an unrelated TAT linked; FITC-tagged peptide had no discernible effect on FTY720-P induced S1P1 receptor internalisation. Results indicate that the extreme c-terminus of the S1P1 receptor is important for the recruitment of unidentified interacting proteins involved in trafficking of the receptor. This may be crucial in coordinating surface expression, receptor recycling and receptor degradation. Interestingly, incubation with the MNP301 (when administered with FTY720-P) served to down regulate receptor expression as seen by reduced receptor fluorescence (**Fig.** 3.11). This observation may be indicative of disruption of sorting signals which would serve to shift sorting preference away from the recycling pathway, and toward the degradation pathway (**Fig.** 1.7). Competitive inhibition by MNP301 of putative phosphatases that target serine residues in the extreme c-terminus of the S1P1 receptor would serve to undermine 'fast' receptor recycling, further disrupting the recycling-to-degradation balance (**Fig.** 3.12). MNP301 presents us with a novel way to modulate interactions occurring at the c-terminus of the S1P1 receptor, providing us with a powerful tool for further research and elucidation of proteins involved in trafficking of the receptor to intracellular compartments.

3.3.3 Possible consequences for astrocytes in MS.

In response to injury, be it inflammatory or physical, astrocytes undergo a change in phenotype referred to as astrogliosis. Astrogliosis is characterised by both antigenic and morphological changes facilitated by increases in cytoskeletal markers such as GFAP, vimentin and nestin. Astrocytes can be categorised phenotypically as follows: (i) Quiescent, as found in normal resting CNS tissue. (ii) Activated, also termed mild gliosis, these astrocytes are thought to help promote myelination in damaged CNS tissue. (iii) Reactive, the most severe phenotypic, and are the astrocytes associated with the glial scar. These cells have been shown to secrete pro-inflammatory cytokines, inhibit remyelination and axonal regeneration. It is important to note that these categories are not mutually exclusive, astrocytic heterogeneity is probably much greater than our current understanding. These cells appear to have the ability to move between these various activation states influenced by their age, various cytokine signals and proximity to sites of injury. In the context of MS, it is plausible that under conditions where local levels of S1P are elevated (i.e. an auto-immune response or lesion) the S1P1 receptor undergoes 'fast' recycling and intense signalling, leading to hyper-activation of astrocytes. This in turn could lead to the development of astrogliosis or a breakdown in astrocytic-endothelial interaction which in-turn could contribute to the 'leaky BBB' phenotype seen in MS. FTY720-P binds to the S1P1 receptor in astrocytes and causes a state of persistent internalisation and eventual degradation as shown in this study. This may 'protect' the cell from progressing to a pathologically reactive state due to continuous activation by excessive local levels of S1P.

3.3.4 Conclusion.

We show that FTY720-P binds to and causes the persistent internalisation of S1P1 receptors in astrocytes. We also show that FTY720-P induced internalisation of the S1P1 receptor can be susceptible to modulation by small molecules (MNP301). The exact mechanism by which this occurs is unknown but we hypothesise that MNP301 competitively inhibits the interaction between the molecular machinery needed for internalisation and the extreme c-terminus of the S1P1 receptor. It would be important to elucidate the interacting proteins involved in this process to further understand the internalisation of the S1P1 receptor in response to FTY720-P or other S1P1 specific compounds. Through the use of MNP301 we have indicated the importance of the extreme c-terminus of the S1P1 receptor for the trafficking of the GPCR to the TGN. However it would be necessary for future work to define the exact key residues that are important for these protein interactions to take place. Taken together these findings provide evidence for the existence of a CNS

target for the novel "immunomodulatory" drug Gilenya®. The significance of this 'central' FTY720-P/S1P1 receptor interaction to the therapeutic action of Gilenya® remains to be seen, however it would be imprudent to dismiss this when discussing Gilenya's therapeutic effect in the treatment of RRMS. Especially when taking into consideration the ease at which this compound gains access to the CNS compartment coupled with the growing body of evidence linking loss of function or gain of aberrant astrocytic function with MS pathology.

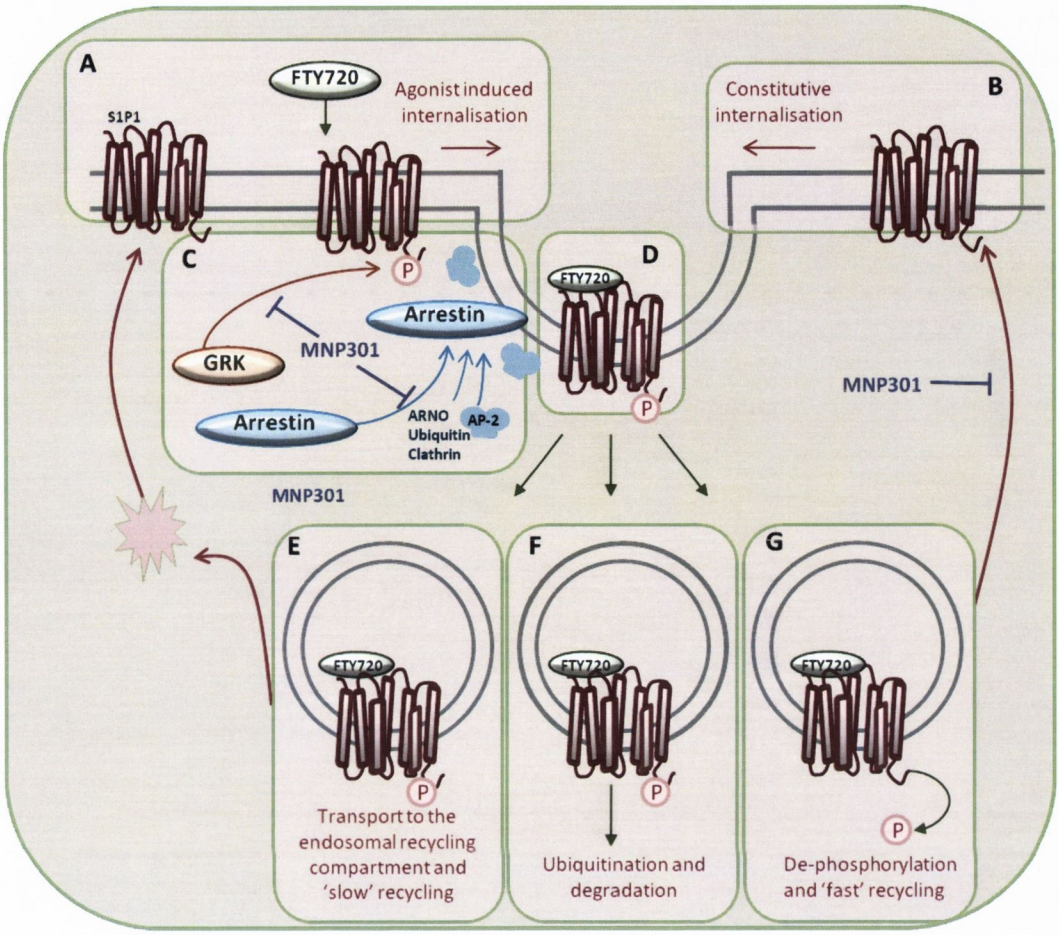


Figure 3.12 *Trafficking of the S1P1 receptor is a multifaceted pathway, with several proposed points of disruption by MNP301.*

(A) Agonist-induced internalisation of the S1P1 receptor. **(B)** Constitutive internalisation of the S1P1 receptor. **(C)** Agonist-induced internalisation occurs as a result of GRK-mediated phosphorylation at the SRR and/or the extreme c-terminus followed by recruitment of adaptor proteins. **(D)** Upon assembly of the clathrin coated pit, the S1P1 receptor is internalised within an early endosome. Early endosomes are subjected to various sorting signals to determine fate of the endosome and its cargo. **(E)** 'Slow' recycling via retrograde transport through the TGN. **(F)** Receptor ubiquitination and degradation in late endosomal/lysosome compartments. **(G)** Dephosphorylation and 'fast' recycling to the surface membrane. Little is known of the potential interactors and endosomal sorting signals for internalised S1P1 receptor at stages **(E)**, **(F)** and **(G)**, though the c-terminus has been implicated as a key site of interaction following observations on the action of MNP301 (purple) reported here.

Chapter 4. The Effect of MNP301 on S1P1 Receptor Downstream Signalling Cascades.

Aims

1: To study the effects of S1P receptor activation on intracellular Ca^{2+} levels in primary rat astrocytes.

2: To investigate the effect of S1P pre-treatment on intracellular Ca^{2+} levels in primary rat astrocytes.

3: To investigate the effect of FTY720-P pre-treatment on intracellular Ca^{2+} levels in primary rat astrocytes.

4: To investigate the effect of AUY954 pre-treatment on intracellular Ca^{2+} levels in primary rat astrocytes.

5: To analyse the effect of MNP301 on S1P1 receptor induced increases in intracellular Ca^{2+} levels in primary rat astrocytes.

6: To study the effect of a unrelated peptide on S1P receptor mediated increases in intracellular Ca^{2+} levels in primary rat astrocytes.

7: To investigate the source of S1P receptor induced intracellular Ca^{2+} in human astrocytes.

8: To investigate the effect of MNP301 on FTY720-P mediated, concentration dependent inhibition of fsk-induced cAMP levels in primary rat astrocytes.

9: To study the effect of MNP301 on FTY720-P induced persistent signalling of S1P receptors in primary rat astrocytes.

Abstract

Gilenya® (Fingolimod; FTY720) is a new oral immunomodulatory therapy for the treatment of RRMS. Its current accepted mode of action is thought to be due to its "functional antagonism" of S1P receptors on autoreactive T-cells in the periphery. However recent work has shown that FTY720-P induced persistently internalised S1P1 receptors continue to signal in a G_i dependent manner, from the TGN and Golgi matrix. S1P1 receptor persistent signalling from intracellular compartments may contribute to Gilenya's therapeutic effect in the treatment of RRMS. Therefore, we aimed to investigate the signalling properties of cell surface expressed and internalised S1P1 receptors in both primary rat astrocytes and human derived astrocytes. Specifically, we focus on Ca^{2+}-dependent and cAMP-dependent downstream signalling pathways. Previously, we have demonstrated that application of MNP301 inhibits FTY720-P induced internalisation of S1P1 receptors to the TGN in primary rat astrocytes. Hence, we investigated the effect of MNP301 on the propagation of downstream signalling. We hypothesised that MNP301 would inhibit FTY720-P induced persistent signalling of S1P1 receptors by preventing receptor trafficking to the TGN. This study also investigated the effect of various S1P receptor agonists on S1P receptor evoked Ca^{2+} signalling events. In addition, the effect of MNP301 on cell surface expressed S1P1 receptor evoked Ca^{2+} signalling was examined. Through the use of various agonists results data showed an abrogation of S1P1 receptor evoked Ca^{2+} signalling upon S1P1 receptor internalisation. In contrast, internalised S1P receptors continued to signal through G_i mediated inhibition of cAMP formation (persistent signalling). Interestingly the data showed that MNP301 completely inhibited S1P1 receptor mediated increases in $_iCa^{2+}$ levels in primary rat astrocytes. The study also showed that S1P1 receptor mediated increases in $_iCa^{2+}$ levels in astrocytes was predominantly due to influx of extracellular Ca^{2+}. Taken together, this data may suggest that putative protein interactions at the c-terminus of the S1P1 receptor are necessary for both receptor internalisation and Ca^{2+} signalling, but not cAMP-dependent signalling in astrocytes.

4.1 Introduction

4.1.1 GPCR persistent signalling.

As described previously, GPCR signalling is thought to involve specific steps, including activation of associated G-proteins and subsequent activation of downstream effector proteins. Following this, most GPCRs are desensitised in a process involving GRK-mediated phosphorylation, binding of β-arrestins and eventual internalisation via either clathrin-coated pits or by other less-defined pathways (Luttrell and Lefkowitz, 2002). Internalisation is thought to serve as a mechanism for both signal termination and a way by which the cell controls GPCR density at the plasma membrane. However recent data suggests that internalisation does not always lead to signal termination. Initial experiments with the β_2-adrenergic receptor and dynamin dominant negative mutants provided evidence that these receptors could associate with components of the ERK pathway in the endosomal compartment (McDonald et al., 2000). Calebiro et al showed that thyroid-stimulating hormone (TSH) bound TSH-receptors are internalised to a "pre-Golgi" compartment where they continue to stimulate production of the second messenger cAMP, in a manner that is distinct from cell membrane bound receptor (Calebiro et al., 2009). Indeed it was also demonstrated that TSH-receptor internalisation and persistent cAMP production was essential for actin rearrangements in thyroid follicle cells (Calebiro et al., 2009). Interestingly, it has been shown that the TSH-receptor can couple to both G_s and $G_{q/11}$ G-proteins (Laugwitz et al., 1996). In fact $G_{q/11}$ mediated signalling pathways are essential for the successful production and release of thyroid hormone (Kero et al., 2007). A recent study has demonstrated that TSH can promote persistent $G_{q/11}$ mediated activation of the phosphoinositide pathway; however this persistent signalling is independent of receptor internalisation (Boutin et al., 2011).

4.1.2 S1P receptor persistent signalling.

Signalling from intracellular compartments has also been demonstrated for lipid sensing GPCRs, as is the case for the lysophosphatidic acid (LPA) receptors. Activation of cell membrane LPA receptors leads to association of $G_{\beta\gamma}$ subunits with the monomeric G-protein Rab11a and subsequent endosomal activation of the Akt signalling pathway which is essential for LPA receptor induced proliferative and anti-apoptotic signals (Garcia-Regalado et al., 2008). Persistent signalling has also been shown to occur in the closely related S1P receptors as demonstrated by the inhibition of cAMP by internalised FTY720-P bound S1P1 receptors (Mullershausen et al., 2009). Data showed that 5hr after removal of FTY720-P, S1P1 receptors continue to exhibit G_i mediated inhibition of forskolin

(fsk) induced cAMP formation; however Ca^{2+} signalling was completely abrogated following receptor internalisation (Mullershausen et al., 2009). This data would suggest that S1P receptor activation of Ca^{2+} mobilising second messengers occurs exclusively at the cell membrane. In contrast it would appear that superagonism of S1P receptors by FTY720-P causes the receptors to be internalised to the TGN/Golgi matrix tightly coupled to components of the adenylyl cyclase pathway, inducing a state of persistent signalling from the intercellular compartment. These studies provide evidence that both internalised and non-internalised GPCRs are capable of persistent signalling to at least three well characterised downstream signalling pathways.

4.1.3 Calcium signalling in astrocytes.

Astrocytes are often considered quiescent compared to neurons, however astrocytes have been shown to express excitability as a result of changes in intracellular calcium levels ($_iCa^{2+}$) (Fiacco and McCarthy, 2006). In a resting state $_iCa^{2+}$ levels are actively maintained between 10-100nM through the actions of a number of voltage- and ligand-gated ion channels, ATPase pumps and ion exchangers found both on organelle outer membranes and the plasma membrane (Fiacco and McCarthy, 2006). These include, Na^+/Ca^{2+} exchangers, plasma membrane Ca^{2+} ATPases (PMCA), transient receptor potential channels (TRPCs) which can be activated by membrane bound DAG, voltage-gated calcium channels (VGCCs) and calcium-permeable AMPA receptors (those lacking the GluR2 subunit) (Ben Achour et al., 2010; Golovina, 2005) (**Fig.** 4.1). Elevations in $_iCa^{2+}$ levels are mainly triggered by agonist binding to G_q coupled GPCRs and subsequent activation of the phospholipase (PLC) pathway, leading to the induction of second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 proceeds to act on its cognate receptor (IP3R) to trigger the release of Ca^{2+} from internal stores (Hatton, 2002). The major intracellular store for Ca^{2+} is the endoplasmic reticulum which acts as natural sink for Ca^{2+} ions. It also been shown that mitochondria can act as an internal Ca^{2+} store in astrocytes due to the expression of various Ca^{2+} channels and transporters such as the mitochondrial Ca^{2+} uniporter (MCU), mitochondrial ryanodine receptor (mRyR) and the mitochondrial Ca^{2+}/H^+ exchanger (LETM1). These proteins allow the mitochondria to either sequester or release Ca^{2+} into the cytosol (Pan et al., 2011; Verkhratsky et al., 2012). One of the principal outcomes of increased cytosolic Ca^{2+} levels in astrocytes is the Ca^{2+}-dependent vesicular release of signalling molecules in a process dubbed 'gliotransmission'. Astrocytes have been shown to release many gliotransmitters, including amino acids such as glutamate and D-serine, nucleotides such as ATP and peptides such as BDNF and atrial natriuretic peptide (Zorec et al., 2012). These chemicals can act upon neighbouring neurons, glia and endothelial cells (Perea and Araque, 2010).

Gliotransmission has been observed to occur via both exocytotic and non-exocytotic release (Kreft et al., 2004). Once released into the extracellular space, these molecules can modulate synaptic transmission and plasticity by acting on neighbouring neurons; they can also affect behaviour through modulation of sleep homeostasis (Parpura and Zorec, 2010; Perea and Araque, 2010). Thus Ca^{2+} signalling in astrocytes is fundamental to synaptic function, with the exact role of S1P receptors yet to be elucidated.

4.1.4 Store operated Ca^{2+} entry.

The major process by which Ca^{2+} gains entry to non-excitable cells is termed, store operated Ca^{2+} entry (SOCE). Emptying of internal Ca^{2+} stores activates Ca^{2+} release activated Ca^{2+} channels (CRAC channels) allowing the influx of Ca^{2+} across the plasma membrane (Parekh and Penner, 1997). CRAC channels of which there are many varying types are the molecular constituents of Ca^{2+}-release activated current (I_{CRAC}). I_{CRAC} is a measurable, non-voltage activated, inwardly rectifying Ca^{2+} current associated with SOCE that was first described in mast cells (Hoth and Penner, 1992). This Ca^{2+} influx appears to be responsible for the major forms of Ca^{2+} dependent signal transduction resulting in both short term cellular responses such as exocytosis and protein-protein interactions and long term responses such as gene transcription (Parekh and Putney, 2005). SOCE is regulated by a dip in ER Ca^{2+} levels irrespective of cytoplasmic Ca^{2+} levels. The sensors that detect organelle Ca^{2+} levels along with the molecular process that governs the relaying of this message to plasma membrane bound CRAC channels is still unknown. SOCE has been identified in both cultured astrocytes and astrocytes in acute rat brain slices, with both Bergmann glia and astrocytes of the granule cell layer shown to exhibit SOCE in cerebellar rat brain slices (Lo et al., 2002; Singaravelu et al., 2006).

4.1.5 S1P receptor mediated Ca^{2+} signalling.

It has been shown that S1P itself can cause the release of Ca^{2+} from intracellular stores, however S1P's ability to increases $_{i}Ca^{2+}$ levels is mainly due to its capacity to act as a ligand for plasma membrane bound S1P receptors (Ghosh et al., 1994; Giussani et al., 2007). S1P agonism of S1P receptors leads to activation of many downstream signalling pathways (**Fig.** 1.5) and is often associated with mobilisation of Ca^{2+} and sphingosine kinase activity (Spiegel and Milstien, 2003; Tas and Koschel, 1998; Young et al., 2000). As described above, GPCR mediated Ca^{2+} mobilisation generally involves G_q induced activation of the PLC/ IP3 pathway. However studies investigating S1P receptor induced increases in $_{i}Ca^{2+}$ levels in astrocytes have indicated a role for G_i coupled S1P1 receptors. It has been shown previously that inhibition with the S1P3 antagonist suramin resulted in

a 60% decrease in S1P mediated $_{i}Ca^{2+}$ rise (Giussani et al., 2007). Conversely inhibition of the S1P1 receptor component by PTX pre-treatment led to an 80% decrease in S1P mediated $_{i}Ca^{2+}$ rise (Giussani et al., 2007). This data indicates that the βγ dimer of the G_i protein is the major component necessary for S1P evoked calcium increases in astrocytes. In agreement with this, studies have also shown that astrocytic IP formation in response to FTY720-P is mainly due to G_i coupled S1P1 receptors with minor involvement of the G_q coupled S1P3 receptor (Mullershausen et al., 2007). Taken together the data suggests that S1P signalling to Ca^{2+} acts via multiple heterotrimeric G-proteins. The source of intracellular calcium in response to S1P stimulation has also been investigated in cerebellar astrocytes. It was observed that S1P administration resulted in a biphasic increase in $_{i}Ca^{2+}$ (Giussani et al., 2007). This is characterised by an initial sharp increase in $_{i}Ca^{2+}$ followed by a sustained increase and slow return to $_{i}Ca^{2+}$ basal levels (Giussani et al., 2007). The data suggests that the initial sharp transient increase in $_{i}Ca^{2+}$ was due to release of Ca^{2+} from intracellular stores, whilst the sustained $_{i}Ca^{2+}$ elevation was due to influx extracellular Ca^{2+} (Giussani et al., 2007).

4.1.5 Chapter Aim.

In the previous chapter we investigated the internalisation properties of agonist bound S1P receptors. In this study we focused on the signalling properties of S1P1 receptors. We aimed to investigate the effect of S1P1 receptor internalisation states on the receptors ability to signal to the AC/cAMP and PLC/IP3 pathways. In addition we studied the effect of the S1P1 receptor blocking peptide MNP301 on S1P1 receptor induced inhibition of cAMP formation and receptor evoked increases in $_{i}Ca^{2+}$ levels. The source of Ca^{2+} involved in S1P1 receptor specific activation remains unidentified and thus was also examined in this study.

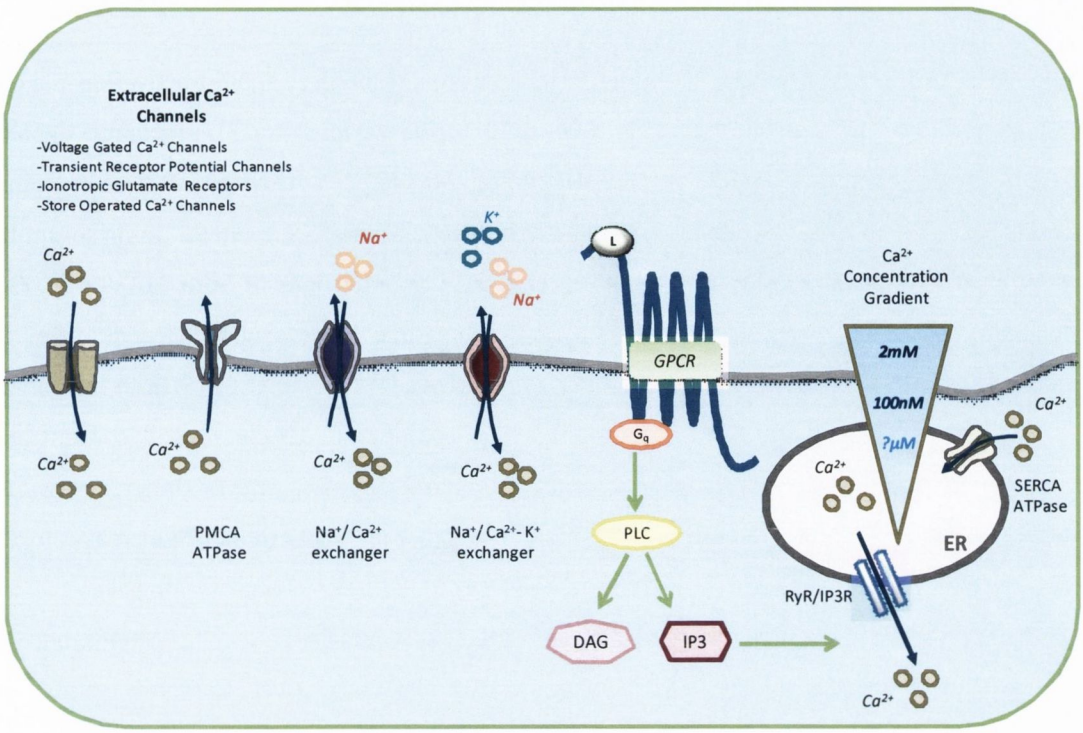


Figure 4.1 *The regulation of intracellular Ca^{2+} levels in astrocytes.*

Schematic depicting how $_{i}Ca^{2+}$ levels are maintained in astrocytes. Plasma membrane Ca^{2+} ATPases (PMCA) pump Ca^{2+} out of the cell while sarcoendoplasmic reticular Ca^{2+} ATPases (SERCA) pump Ca^{2+} into the ER. ATPases function by exchanging protons for one (PMCA) or two (SERCA) Ca^{2+} ions per ATP hydrolysed. In addition membrane bound antiporter proteins exchange one Ca^{2+} ion for three Na^{+} ions (Na^{+}/ Ca^{2+} exchanger) or co-transport one Ca^{2+} ion and one K^{+} ion for four Na^{+} ions. This combination of pumps and exchangers help establish a Ca^{2+} concentration gradient, keeping resting state Ca^{2+} between 10-100nM, roughly 10,000-20,000 times lower than that of extracellular Ca^{2+} . G_q mediated activation of the PLC/IP3 pathway is the most accepted mechanism for increases in $_{i}Ca^{2+}$ levels. IP3 binds to and activates its cognate receptor on the surface of the ER resulting in the release of Ca^{2+} into the cytosol. However Ca^{2+} can also gain access to astrocytes through a number of plasma membrane bound receptors. These include AMPA receptors lacking the GluR2 subunit, the non-selective Ca^{2+} permeable transient receptor potential channels (TRPCs) and voltage gated Ca^{2+} channels.

4.2.2 S1P receptor agonists cause a concentration dependent increase in $_{i}Ca^{2+}$ levels in primary rat astrocytes.

It has been shown previously that S1P receptors in astrocytes respond to agonist activation in a Ca^{2+}-dependent manner (Mullershausen et al., 2007). To further investigate S1P receptor signalling events in astrocytes, the ability of both S1P (**Fig.** 4.2A) and the S1P1 receptor selective compound AUY954 (**Fig.** 4.2B) to elicit Ca^{2+} signals was examined. Astrocytes were starved for 3hr in serum-free media prior to stimulation with 1μM, 100nM and 10nM concentrations of both S1P and AUY954. Changes in $_{i}Ca^{2+}$ levels were recorded over a time course of 240secs; following addition of test compound for 150secs, glutamate was added for a further 60secs. Treatment with both the pan-S1P receptor agonist S1P and the S1P1 receptor selective compound AUY954 caused concentration dependent increases in $_{i}Ca^{2+}$ levels in primary rat astrocytes (**Fig.** 4.2A and B; Upper panels). S1P activates all five S1P receptors and therefore it is thought that its ability to induce increases in $_{i}Ca^{2+}$ levels is predominantly due to its activation of the G_q-coupled S1P3 receptors. However the data confirmed that AUY954 elicits Ca^{2+} signalling in astrocytes through its specific actions on S1P1 receptors. This finding is intriguing as S1P1 receptors are coupled exclusively to G_i. It is possible that under certain circumstances or in certain cell types S1P1 receptors can couple to G_q, however it is more likely that S1P1 receptor mediated Ca^{2+} signals are due to the known ability of $G_{\beta\gamma}$ subunits to activate PLC (Birnbaumer, 1992).

4.2.3 S1P pre-treatment inhibits S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in rat astrocytes.

To examine the effect of receptor internalisation states on S1P receptors ability to signal in a Ca^{2+}-dependent manner, primary rat astrocytes were pre-treated with various S1P receptor agonists. Serum starved cells were pre-treated with 1μM S1P for 1hr followed by a 3hr washout period to ensure $_{i}Ca^{2+}$ levels returned to baseline prior to stimulation. The data shows that S1P (1μM) pre-treatment significantly reduced the cell's ability to respond to further stimulation by S1P (**Fig.** 4.3A) and AUY954 (**Fig.** 4.3B) (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test). This effect is most likely due to saturation of common S1P receptors by the high concentration of S1P rather than depletion of Ca^{2+} stores *per se*. This is in agreement with the cells responding to subsequent application of glutamate (1μM) (**Fig.** 4.3A and B; Upper panels). The reduced effect is likely caused by recycled S1P receptors that have been stripped of agonist and recycled back to the cell surface through the endosomal recycling compartment.

4.2.4 Pre-treatment with FTY720-P inhibits S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in rat astrocytes.

Previously we have shown that FTY720-P treatment causes sustained internalisation of S1P1 receptors in primary rat astrocytes even after a 5hr washout of the drug (**Fig.** 3.5). Thus we examined the effect of FTY720-P induced internalisation of S1P receptors on these receptors ability to elicit Ca^{2+} signals. Cells were pre-treated with 1μM FTY720-P for 1hr followed by a 3hr washout period. Pre-treatment of astrocytes with FTY720-P prevented astrocytes from responding to subsequent stimulation with the S1P1 receptor selective compound AUY954 (**Fig.** 4.4B). This is likely due to complete internalisation of the S1P1 receptor by FTY720-P, leading to a complete loss from the cell surface, therefore leaving no receptor available to respond to AUY954. Astrocytes pre-treated with FTY720-P had a significantly reduced response to stimulation with 1μM S1P (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test) (**Fig.** 4.4A). The data suggests that this reduced response may be due to FTY720-P induced partial agonism of S1P3 receptors. In other words, surface expressed G_q-coupled S1P3 receptors that have not been internalised by FTY720-P are capable of responding to S1P.

4.2.5 Pre-treatment with the S1P1 receptor specific agonist AUY954 inhibits S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in rat astrocytes.

Next, we investigated the relative of contribution of S1P1 receptors to S1P receptor mediated elevations in $_{i}Ca^{2+}$ levels. To specifically evaluate S1P1 receptors, primary rat astrocytes were pre-treated with 1μM AUY954 for 1hr, followed by a 3hr washout period. Cells were subsequently stimulated with S1P (1μM) (**Fig.** 4.5A) and AUY954 (1μM) (**Fig.** 4.5B). The data showed that AUY954 pre-treatment significantly attenuated astrocytes response to S1P (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test) (**Fig.** 4.5A; Lower panel). As expected, pre-treatment with AUY954 completely abrogated astrocytes Ca^{2+} response to stimulation with 1μM AUY954 (**Fig.** 4.5B). Again this is most likely due to the complete internalisation of cell surface S1P1 receptors, rendering the cell incapable of responding to extracellular activation. The results can be explained by S1P promoting increases in $_{i}Ca^{2+}$ levels via S1P1 and S1P3 receptors. This result would suggest that S1P1 receptors contribute to approximately 50% of the observed Ca^{2+} response to S1P stimulation. As seen when S1P1 receptors are internalised by AUY954 pre-treatment, the remaining Ca^{2+} response observed when cells are stimulated with S1P would constitute the S1P3 evoked portion of the response.

4.2.6 MNP301 specifically antagonises S1P1 receptor mediated increases in $_{i}Ca^{2+}$ levels in rat astrocytes.

Having previously shown that MNP301 prevented FTY720-P induced S1P1 receptor internalisation to the TGN (**Fig.** 3.10), its effect on S1P1 receptor mediated Ca^{2+} signalling was examined. Primary rat astrocytes were incubated with 100μg/ml concentration of MNP301 for 1hr followed by a 3hr washout period. Cells were then stimulated with 1μM S1P (**Fig.** 4.6A) and 1μM AUY954 (**Fig.** 4.6B). MNP301 significantly reduced S1P induced increase in $_{i}Ca^{2+}$ levels in astrocytes (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test) (**Fig.** 4.6A; Lower panel). Stimulation of MNP301 pre-treated astrocytes with the S1P1 selective compound AUY954 showed that MNP301 completely attenuated the S1P1 receptor specific increase in $_{i}Ca^{2+}$ levels (**Fig.** 4.6B). This antagonistic effect of the MNP301 peptide may be due to interference of interactions between adaptor proteins or second messenger proteins necessary for the propagation of Ca^{2+} signals at the extreme c-terminus of the S1P1 receptor.

4.2.7 Pre-treatment with unrelated peptide MNP201 does not affect S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in rat astrocytes.

Similar to S1P1 receptor internalisation experiments (**Fig.** 3.10) the selective effect of MNP301 on S1P1 receptor induced Ca^{2+} signalling was investigated. Primary rat astrocytes were treated for 1hr with 100μg/ml MNP201 (FITC-Ahx-YGRKKRRQRRRVCMGDHWFDV), a TAT-based, FITC tagged unrelated peptide. Following a 3hr washout period, cells were stimulated with 1μM S1P. The data showed that MNP201 had no effect on S1P induced Ca^{2+} signalling in rat astrocytes (**Fig.** 4.7). Taken together this result indicated that MNP301 inhibited S1P1 receptor induced Ca^{2+} signalling in a selective manner which was not dependent on the fused TAT sequence, as a unrelated peptide, MNP201 had no observed effect on S1P receptor induced Ca^{2+} signalling events.

4.2.8 S1P receptor agonists induce a concentration dependent increase in $_{i}Ca^{2+}$ levels in human astrocytes.

To evaluate the source of $_{i}Ca^{2+}$ in astrocytes stimulated with S1P receptor agonists the effect of both antagonists of IP3/ryanodine receptors (dantrolene) and chelators of extracellular Ca^{2+} (EGTA) on S1P receptor induced elevations of $_{i}Ca^{2+}$ were examined. These studies were performed using human astrocytes expressing endogenous S1P receptors (**Fig.** 4.8A; Upper panels). The effect of both S1P (**Fig.** 4.8B; Lower panels) and the S1P1 specific compound SEW2871 (**Fig.** 4.8C; Lower panels) on $_{i}Ca^{2+}$ levels in human astrocytes was first examined. Data showed that both S1P (*** $p<0.001$, 100nM and

1μM vs. control, one-way ANOVA and Bonferroni post-hoc test) and SEW2871 (*** p<0.001, 1μM and 10μM vs. control, as determined by unpaired t-test) induced a concentration dependent increase in $_{i}Ca^{2+}$ levels. S1P induced higher levels of $_{i}Ca^{2+}$, likely due to activation of all five receptors versus SEW2871's selective activation of S1P1 receptors (**Fig.** 4.8B and C).

4.2.9 S1P receptor mediated increase in $_{i}Ca^{2+}$ is from a predominantly extracellular source.

It has been previously shown that S1P receptor evoked Ca^{2+} signalling in astrocytes was due to activation of both intracellular stores and entry of extracellular Ca^{2+} to the cell (Giussani et al., 2007). However the source of $_{i}Ca^{2+}$ in response to S1P1 receptor specific activation remains unclear. The involvement of Ca^{2+} released from intracellular stores was evaluated by inhibiting IP3/ryanodine receptors that are involved in the release of Ca^{2+} from the ER. Cells were pre-treated with 30μM dantrolene for 10mins. Cells were subsequently stimulated with 1μM S1P dissolved in both Ca^{2+} containing HBSS and HBSS minus Ca^{2+}. Cells pre-treated with 30μM dantrolene (+Ca^{2+}) showed a slow increase in $_{i}Ca^{2+}$ levels, peaking at 90secs post SEW2871 stimulation (**Fig.** 4.9A). Cells pre-treated with 30μM dantrolene (-Ca^{2+}) a relatively slower Ca^{2+} signalling event was observed, with cells only beginning to respond ~80secs post SEW2871 stimulation (**Fig.** 4.9A). Both dantrolene treatments, while not completely inhibiting elevations in $_{i}Ca^{2+}$ levels, significantly reduced S1P1 receptor mediated Ca^{2+} responses (*** p<0.001, dantrolene (+Ca^{2+}) and dantrolene (-Ca^{2+}) vs. control, one-way ANOVA and Dunnetts post-hoc test). To examine the contribution of extracellular sourced Ca^{2+} in S1P1 receptor evoked Ca^{2+} signalling, cells were pre-treated with 1mM EGTA for 10mins followed by stimulation with SEW2871 dissolved in HBSS minus Ca^{2+}. Data showed that cells failed to respond to SEW2871 stimulation in the presence of the extracellular chelating agent EGTA (**Fig.** 4.8A). EGTA pre-treatment significantly inhibited S1P1 receptor mediated increase in $_{i}Ca^{2+}$ levels. We therefore conclude that S1P1 receptor evoked Ca^{2+} signalling utilises predominantly Ca^{2+} from an extracellular source. However a ~50% increase in baseline Ca^{2+} levels was observed (**Fig.** 4.9A; Upper panel), indicating some involvement of intracellularly stored Ca^{2+}.

4.2.10 MNP301 treatment does not alter FTY720-P's dose dependent inhibition of fsk-induced cAMP formation in primary rat astrocytes.

In addition to Ca^{2+} signalling, it is known that S1P receptors signal in a cAMP-dependent manner (Mullershausen et al., 2007). Previous studies, using HUVECs showed that treatment with FTY720-P followed by a 5hr washout period induced a persistent internalisation of S1P1 receptors and a state of persistent signalling as indicated by inhibition of cAMP production (Mullershausen et al., 2009). In

addition we examined the effect of MNP031 on the dose-response relationship of FTY720-P on cAMP formation. Cells were starved in serum-free medium prior to all experiments and then treated with increasing concentrations of FTY720-P for 20min in the absence and presence of a 1hr pre-incubation with MNP301. Addition of MNP301 did not cause a shift in the dose-response and the maximal response remained unchanged. In addition pre-incubation with MNP301 did not alter the inhibitory concentration for half maximum response (IC_{50}) (**Fig.** 4.10). Increasing concentrations of FTY720-P (20min treatment) generated IC_{50} values for fsk-induced cAMP formation of 0.07 +/- 0.24nM and 0.05 +/- 0.14nM with and without pre-treatment of MNP301 (100μg/ml for 1hr), respectively (**Fig.** 4.10). Therefore we conclude that while MNP301 might alter the internalisation of the S1P1 receptor and S1P1 receptor evoked Ca^{2+} signalling but does not affect FTY720-P mediated signalling to cAMP.

4.2.11 MNP301 does not alter FTY720-P induced persistent signalling of S1P receptors.

Next we determined whether the FTY720-P internalised pool of S1P1 receptors in astrocytes had the ability to persistently inhibit fsk-induced cAMP formation after washout of the drug. The aim of this experiment was to determine whether FTY720-P internalised S1P1 receptors signalled persistently from inside the astrocytes as was observed in HUVECs (Mullershausen et al., 2009), in addition to investigating the effect of MNP301 on persistent S1P1 receptor signalling. We hypothesized that by preventing the S1P1 receptor from reaching the peri-nuclear region we might prevent persistent signalling. The data showed that FTY720-P (1μM for 20mins) attenuated fsk-induced cAMP formation (42.1% +/- 4.8 inhibition) (**Fig.** 4.11, lane 2) compared to forskolin alone (**Fig.** 4.11, lane 1). Fsk-induced cAMP formation remained markedly attenuated in astrocytes treated with FTY720-P (1μM for 1hr) even 5hr after washout of the drug (69.7% +/- 5.2 inhibition) (**Fig.** 4.11, lane 3), thus providing evidence for continued signalling of the internalised receptor. To differentiate between the effects of FTY720-P on surface- and internalised-S1P1 receptors, its effects on fsk-induced cAMP formation were determined in the presence of MNP301. The data showed that despite attenuation of S1P1 receptor internalisation by FTY720-P (**Fig.** 4.11), the persistent signalling (persistent cAMP inhibition) caused by FTY720-P treatment (1μM for 1hr followed by 5hr washout) was not altered by pre-treatment of MNP301 (100μg/ml for 1hr) (65.7% +/-3.3 inhibition) (**Fig.** 4.11, lane 4). Collectively, we conclude that while MNP301 prevents FTY720-P induced internalisation of S1P1 receptors and Ca^{2+} signalling, the peptide does not affect persistent signalling of these receptors to the cAMP pathway.

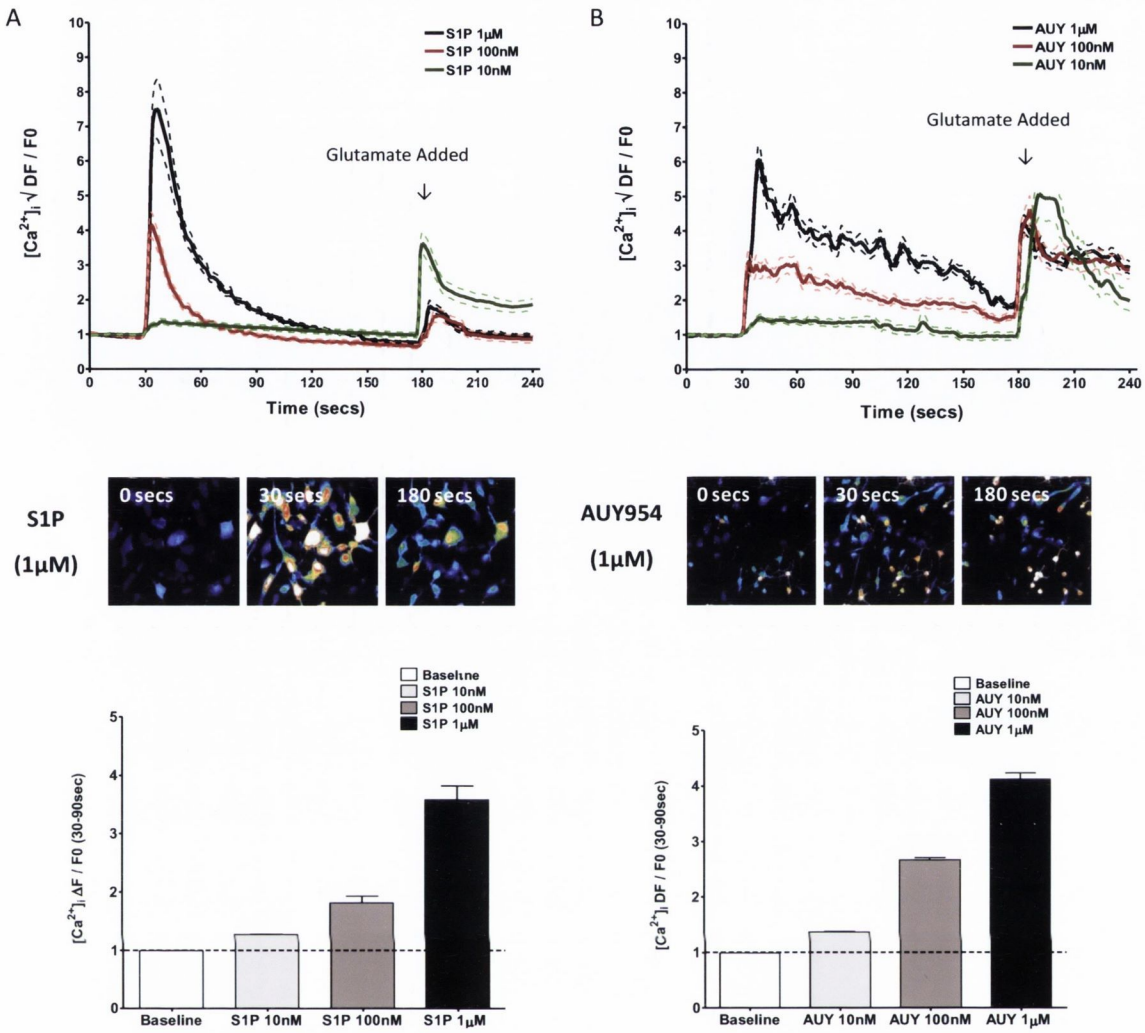


Figure 4.2 *S1P receptor agonists induce a concentration dependent increase in $_{i}Ca^{2+}$ levels in primary rat astrocytes.*

Increasing concentrations of (**A**) the natural ligand S1P and (**B**) the S1P1 specific agonist AUY954 induce a concentration dependent increase in $_{i}Ca^{2+}$ levels. Upper panels show traces depicting changes in $_{i}Ca^{2+}$ levels over time. Lower panels, corresponding bar graphs (30–90 secs) show that S1P and AUY954 cause a concentration dependent increase in $_{i}Ca^{2+}$ levels. Middle panels, representative images were taken from time-lapse series, before addition of S1P/AUY954 (0secs), after addition of 1μM S1P/AUY954 (30secs) and after stimulation with 1mM glutamate (180secs). Data represented as mean ± SEM. n=2, all conditions were carried out in quadruplicate. (Cells counted, S1P 1μM=200, S1P 100nM=185, S1P 10nM=132; AUY 1μM=235, AUY 100nM=171, AUY 10nM=85).

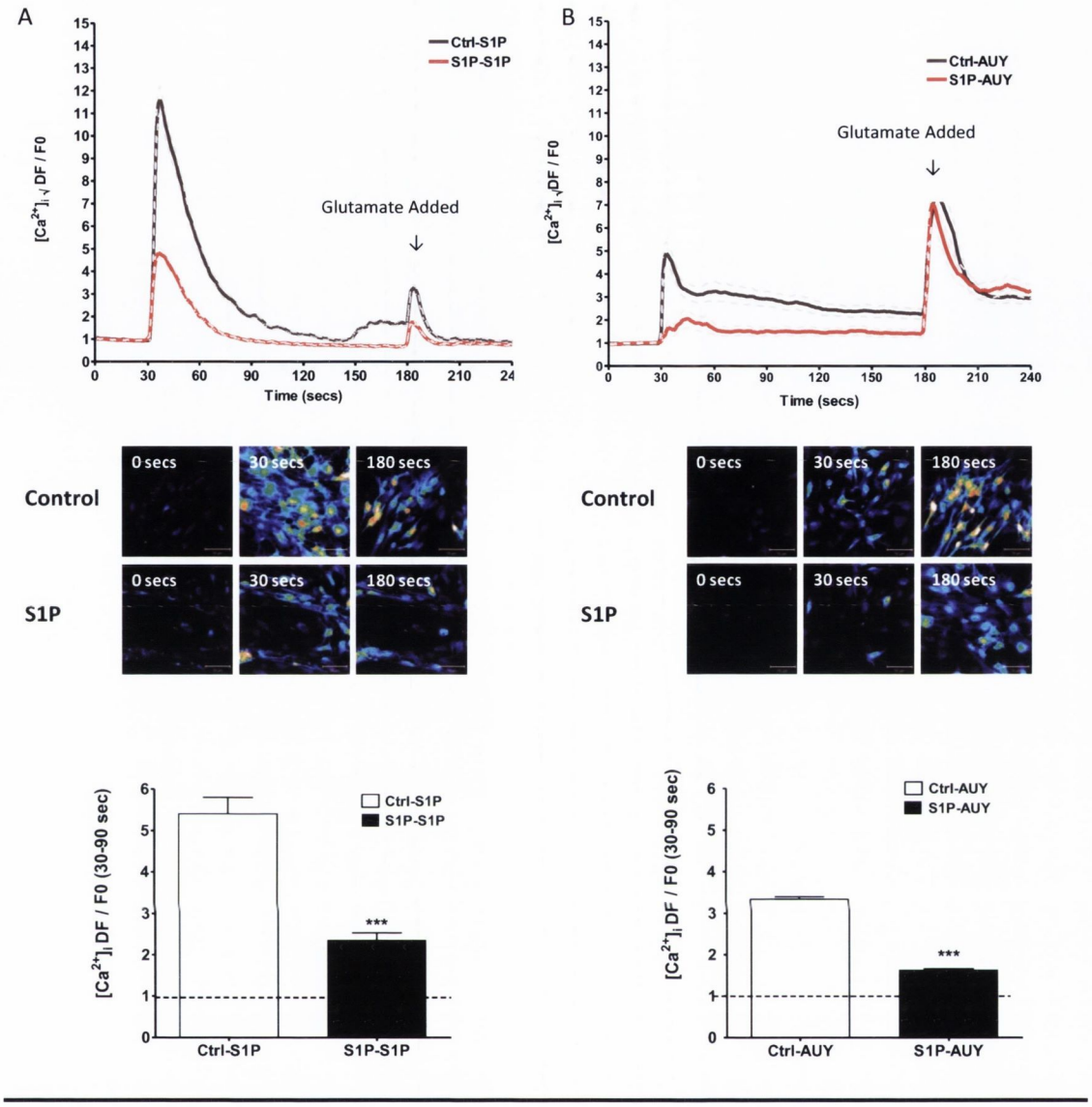


Figure 4.3 *S1P pre-treatment inhibits S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in astrocytes.*

S1P pre-treatment reduced astrocyte Ca^{2+} responses to stimulation with both (**A**) S1P and (**B**) AUY954. Upper panels show traces depicting changes in $_{i}Ca^{2+}$ levels over time. Lower panels, corresponding bar graphs (30–90secs) show that S1P significantly inhibited changes in $_{i}Ca^{2+}$ levels in response to both S1P and AUY954 (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test). Middle panels, representative images were taken from time-lapse series, before addition of S1P/AUY954 (0secs), after addition of 1μM S1P/AUY954 (30secs) and after stimulation with 1mM glutamate (180secs). Data represented as mean ± SEM. n=2, all conditions were carried out in quadruplicate. (Cells counted, Ctrl-S1P=648, S1P-S1P=746; Ctrl-AUY=479, S1P-AUY=446).

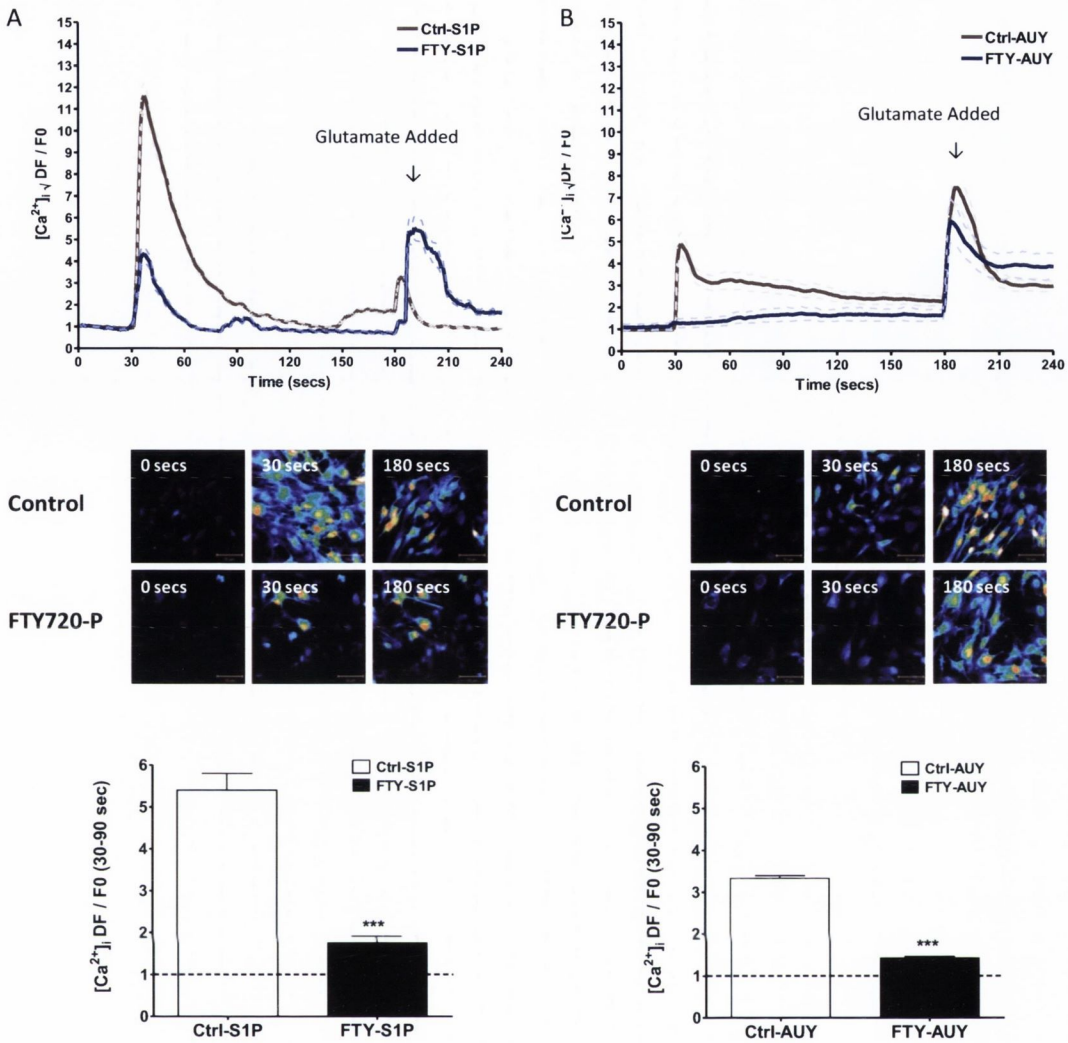


Figure 4.4 *Pre-treatment with FTY720-P inhibits S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in astrocytes.*

FTY720-P pre-treatment reduced astrocyte Ca^{2+} responses to stimulation with (**A**) S1P and completely inhibited (**B**) AUY954 induced changes in $_{i}Ca^{2+}$ levels. Upper panels show traces depicting changes in $_{i}Ca^{2+}$ levels over time. Lower panels, corresponding bar graphs (30–90secs) show that S1P significantly inhibited changes in $_{i}Ca^{2+}$ levels in response to both S1P and AUY954 (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test). Middle panels, representative images were taken from time-lapse series, before addition of S1P/AUY954 (0secs), after addition of 1μM S1P/AUY954 (30secs) and after stimulation with 1mM glutamate (180secs). Data represented as mean ± SEM. n=2, all conditions were carried out in quadruplicate. (Cells counted, Ctrl-S1P=648, FTY-S1P=172; Ctrl-AUY=479, FTY-AUY=502).

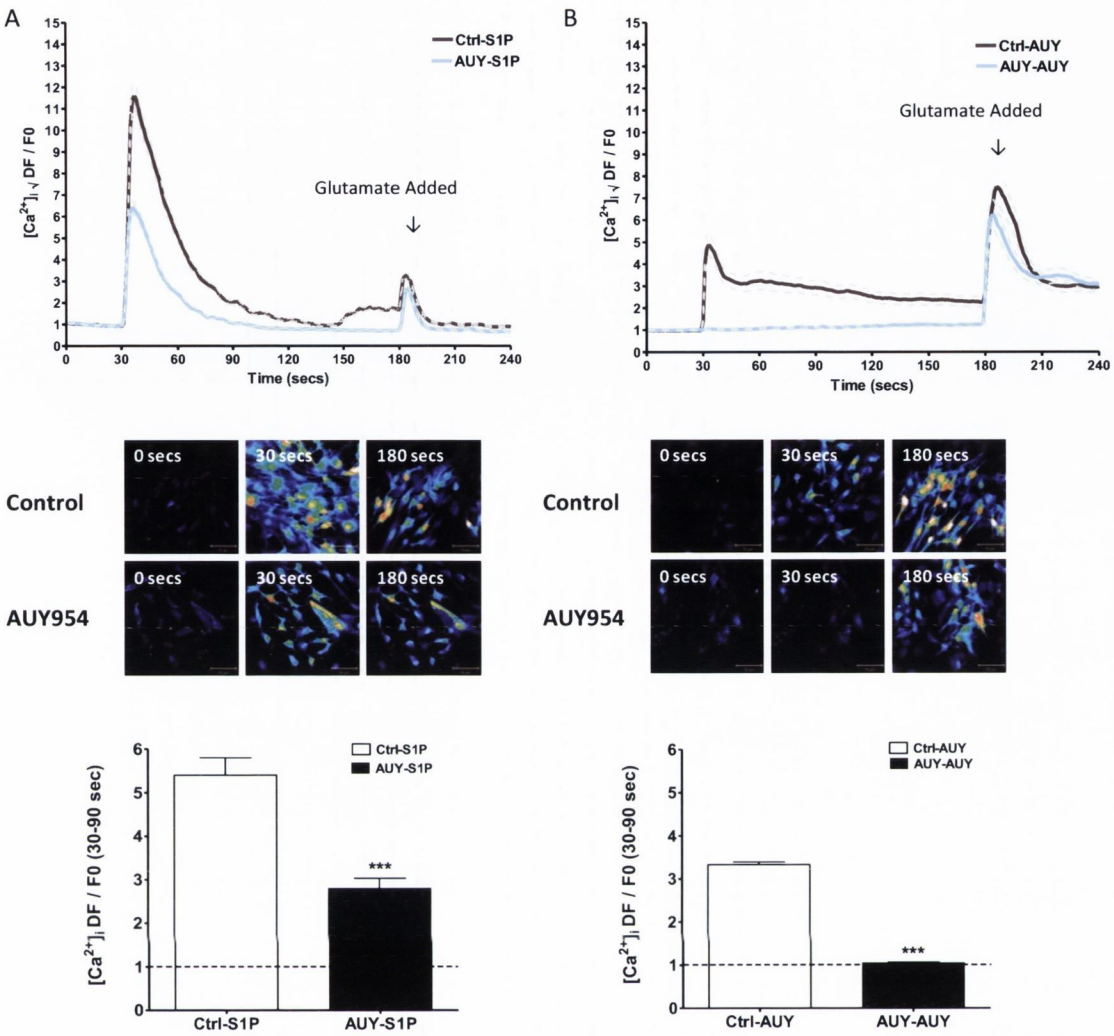


Figure 4.5 *Pre-treatment with the S1P1 receptor specific agonist AUY954 inhibits S1P receptor mediated increases in $_{i}Ca^{2+}$ levels in astrocytes.*

AUY954 pre-treatment reduced astrocytes response to (**A**) S1P and inhibited (**B**) AUY954 induced increases in $_{i}Ca^{2+}$ levels. Upper panels show traces depicting changes in $_{i}Ca^{2+}$ levels over time. Lower panels, corresponding bar graphs (30–90secs) show that S1P significantly inhibited changes in $_{i}Ca^{2+}$ levels in response to both S1P and AUY954 (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test). Middle panels, representative images were taken from time-lapse series, before addition of S1P/AUY954 (0secs), after addition of 1μM S1P/AUY954 (30secs) and after stimulation with 1mM glutamate (180secs). Data represented as mean ± SEM. n=2, all conditions were carried out in quadruplicate. (Cells counted, Ctrl-S1P=648, AUY-S1P=481; Ctrl-AUY=479, AUY-AUY=401).

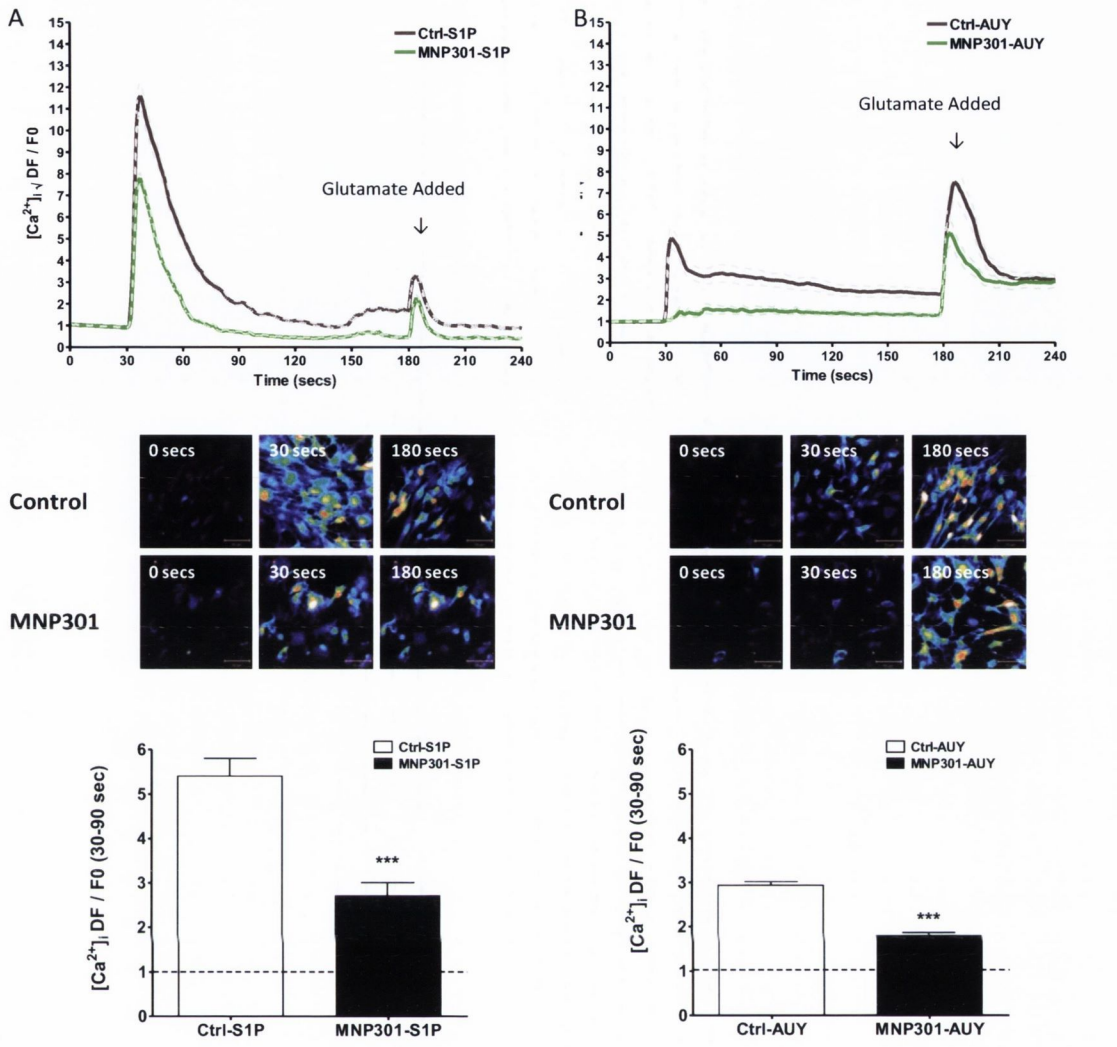


Figure 4.6 *MNP301 specifically antagonises S1P1 receptor mediated increases in $_{i}Ca^{2+}$ levels in astrocytes.*

MNP301 pre-treatment reduced the response of astrocytes to (**A**) S1P and completely inhibited (**B**) AUY954 induced increase in $_{i}Ca^{2+}$ levels. Upper panels show traces depicting changes in $_{i}Ca^{2+}$ levels over time. Lower panels, corresponding bar graphs (30–90secs) show that S1P significantly inhibited changes in $_{i}Ca^{2+}$ levels in response to both S1P and AUY954 (*** $p<0.001$ pre-treatment vs. control, as determined by unpaired t-test). Middle panels, representative images were taken from time-lapse series, before addition of S1P/AUY954 (0secs), after addition of 1μM S1P/AUY954 (30secs) and after stimulation with 1mM glutamate (180secs). Data represented as mean ± SEM. n=2, all conditions were carried out in quadruplicate. (Cells counted, Ctrl-S1P=648, MNP301-S1P=612; Ctrl-AUY=479, MNP301-AUY=399).

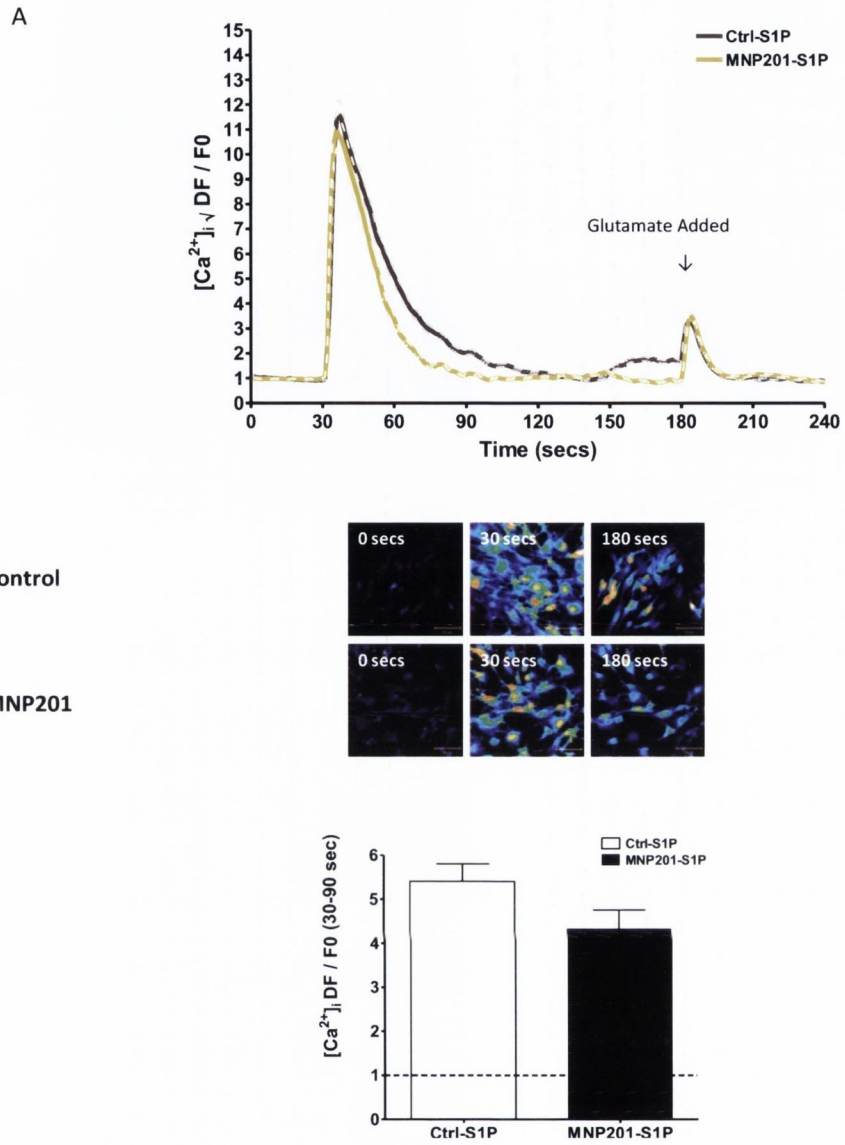


Figure 4.7 *Unrelated peptide MNP201 pre-treatment does not affect S1P receptor mediated increases in $_iCa^{2+}$ levels in astrocytes.*

MNP201 pre-treatment had no effect on (**A**) S1P evoked increases in $_iCa^{2+}$ levels in primary rat astrocytes. Lower panel, corresponding bar graphs (30–90secs) show that MNP201 had no significant effect on S1P induced changes in $_iCa^{2+}$ levels. Middle panels, representative images were taken from time-lapse series, before addition of S1P (0secs), after addition of 1μM S1P (30secs) and after stimulation with 1mM glutamate (180secs). Data represented as mean ± SEM. n=2, all conditions were carried out in quadruplicate. (Cells counted, Ctrl-S1P=648, MNP201-S1P=799).

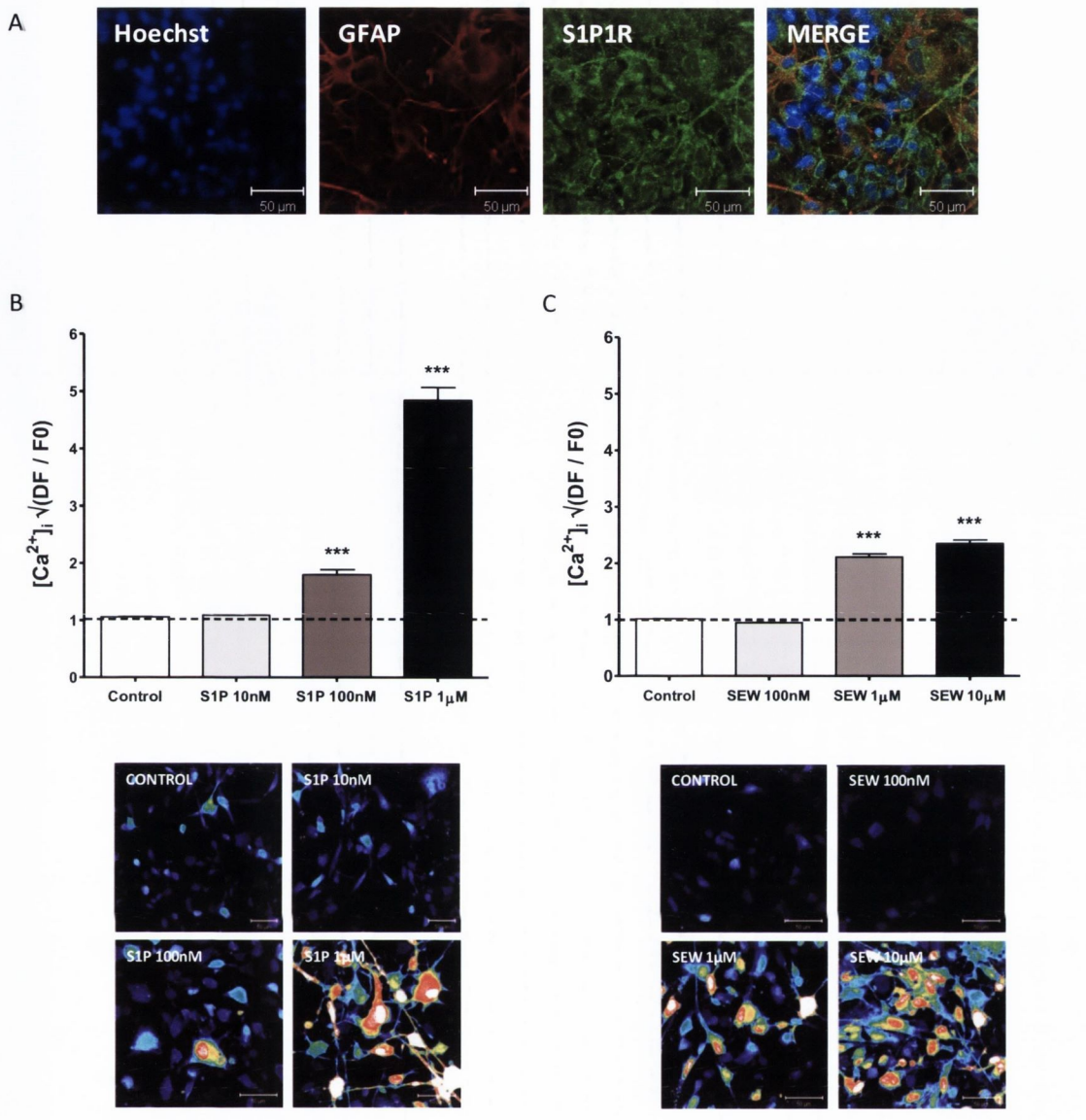


Figure 4.8 *Agonism of S1P receptors evokes increases in $_{i}Ca^{2+}$ levels in human astrocytes.*

Both (**B**) S1P and (**C**) the S1P1 specific compound SEW2871 induced a concentration dependent increase in $_{i}Ca^{2+}$ levels in human astrocytes. Middle panels, bar graphs (30–90secs) show that S1P (*** p<0.001, 100nM and 1µM vs. control, one-way ANOVA and Bonferroni post-hoc test) and SEW2871 (*** p<0.001, 1µM and 10µM vs. control, one-way ANOVA and Bonferroni post-hoc test) significantly increased levels of $_{i}Ca^{2+}$. Lower panels, representative images were taken from time-lapse series, after addition of increasing concentrations of S1P and SEW2871 (30secs). Top panel, immunocytochemistry shows endogenous expression of S1P1 receptors (green) in human astrocytes stained for GFAP (red). n=3, scale bars 50µm.

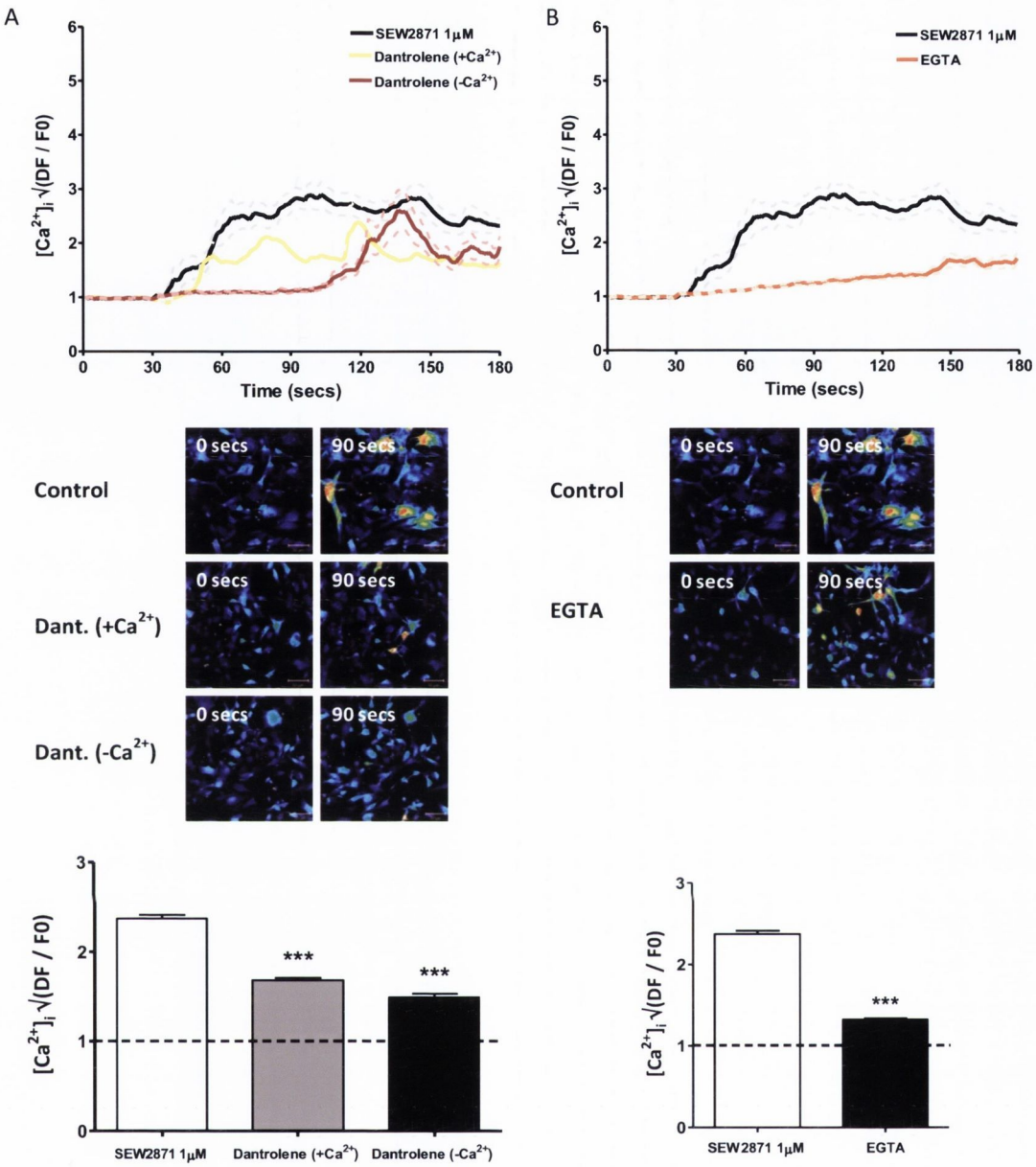


Figure 4.9 *S1P1 receptor mediated increase in $_{i}Ca^{2+}$ is from a predominantly extracellular source.* Pre-treatment of human astrocytes with (**A**) 30μM dantrolene, both in the presence and absence of Ca^{2+} significantly reduced cells response to 1μM SEW2871 (*** $p<0.001$ dantrolene vs. SEW2871 one-way ANOVA, Dunnetts post-hoc test). Pre-treatment with (**B**) 1mM EGTA significantly inhibited S1P1 induced increases in $_{i}Ca^{2+}$ levels, with a 50% increase in basal Ca^{2+} observed (*** $p<0.001$ EGTA vs. SEW2871, unpaired t-test). Middle panels, representative images were taken before (0secs) and after addition of SEW2871 (90secs). n=3, scale bars 50μm.

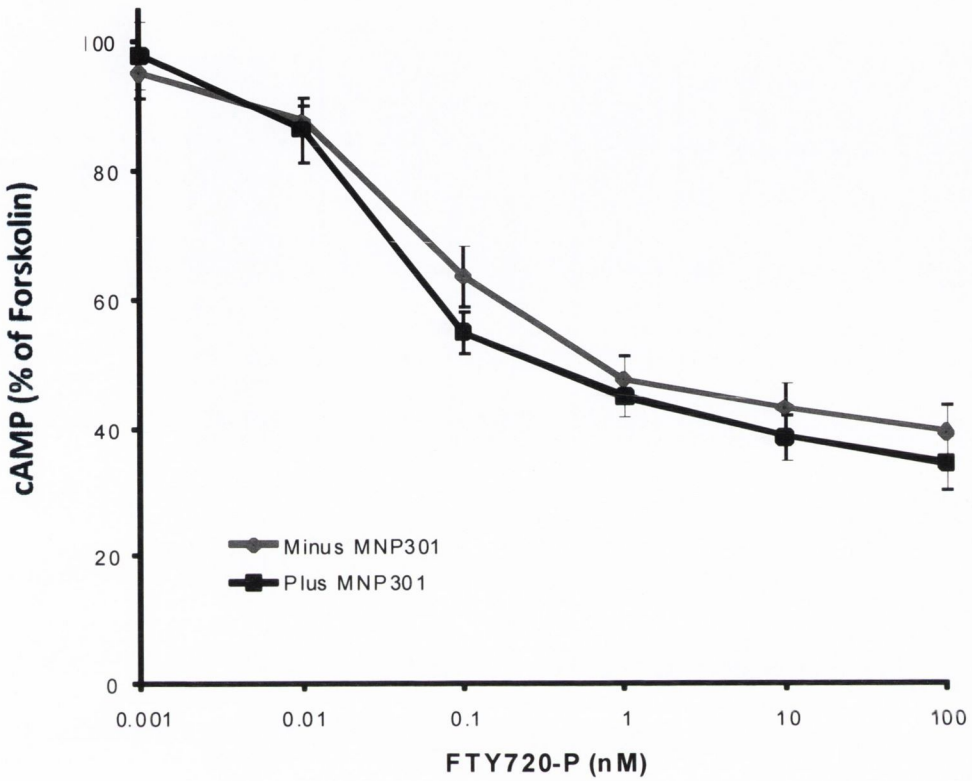
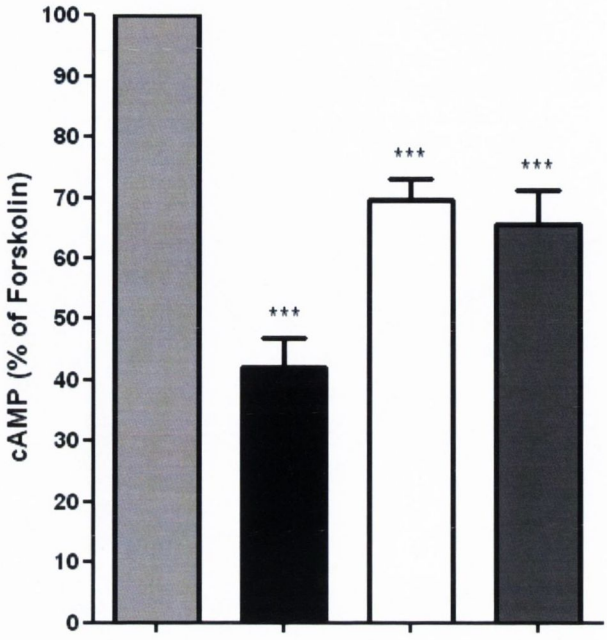


Figure 4.10 *MNP301 treatment does not alter FTY720-P's dose dependent inhibition of fsk-induced cAMP formation in primary rat astrocytes.*

Treatment of primary rat astrocytes with increasing concentrations (0.001nM – 100nM) of FTY720-P caused concentration dependent inhibition of fsk-induced cAMP. Pre-treatment with 100µg/ml MNP301 for 1hr followed by stimulation with increasing concentrations of FTY720-P had no significant effect on FTY720-P induced inhibition of fsk-induced cAMP levels. Data presented as percentage cAMP inhibition, ± SEM, n=3.



4.11 *MNP301 does not alter FTY720-P induced persistent signalling of S1P receptors.*

Percentage inhibition of fsk-induced cAMP formation in a primary rat astrocyte culture showed that 100nM FTY720-P significantly inhibited fsk-induced cAMP formation (*** $p<0.001$, 1μM FTY720-P vs. fsk alone, one-way ANOVA and Bonferroni post-hoc test). S1P receptors continued to inhibit fsk-induced cAMP after pre-treatment with 1μM FTY720-P, followed by a 5hr washout period (persistent signalling) (*** $p<0.001$, 1μM FTY720-P pre-treatment vs. fsk alone, one-way ANOVA and Bonferroni post-hoc test). Cells pre-incubated with 100μg/ml MNP301 followed by a 1μM FTY720-P treatment and 5hr washout period showed that MNP301 had no significant effect on S1P receptor mediated persistent inhibition of fsk-induced cAMP formation. Data presented as percentage cAMP inhibition, ± SEM, n=3.

4.3 Discussion

In this study two S1P1 receptor downstream signalling pathways, namely the AC/cAMP pathway and the PLC/IP3 Ca^{2+} dependent pathway were examined. Both S1P and AUY954 induced a concentration dependent increase in $_{i}Ca^{2+}$ levels, with S1P being a more potent activator most likely due to the agonism of the G_q coupled S1P3 in addition to the exclusively G_i coupled S1P1 receptor (**Fig.** 4.1). The effect of a number of S1P agonists on S1P and AUY954 evoked increases in $_{i}Ca^{2+}$ levels were also examined. In agreement with a previous study the data showed that S1P pre-treatment decreases astrocytic Ca^{2+} responses to further stimulation with both S1P and AUY954. This is likely due to saturation of S1P receptors with some receptors undergoing 'fast' recycling back to the plasma membrane, contributing to the observed reduced response. In contrast pre-treatment with FTY720-P completely abolished further AUY954 evoked Ca^{2+} responses in astrocytes, due to complete internalisation of the S1P1 receptor to the TGN as seen previously (**Fig.** 3.5). Astrocytes pre-treated with FTY720-P exhibited a small Ca^{2+} signal in response to stimulation to S1P. This is most likely due to FTY720-P mediated partial agonism of S1P3 receptors, resulting in a portion of S1P3 receptors remaining at the cell surface to respond to S1P. Pre-treatment with the S1P1 specific compound AUY954 partially inhibited S1P evoked Ca^{2+} signals due to functional antagonism of S1P1 receptors. As expected AUY954 pre-treatment completely abrogated subsequent AUY954 mediated increases in $_{i}Ca^{2+}$ levels due to the lack of S1P1 receptors at the cell surface. The data also showed that MNP301 pre-treatment specifically inhibits the S1P1 portion of S1P induced Ca^{2+} response in astrocytes. This was confirmed by showing that the peptide completely attenuated AUY954 evoked Ca^{2+} signalling. The specificity of this effect was demonstrated by data showing that MNP201, a non-related, TAT tagged, FITC labelled peptide had no measurable effect on S1P induced Ca^{2+} signalling. Next, the source of $_{i}Ca^{2+}$ in human astrocytes was investigated. Data showed that astrocytic $_{i}Ca^{2+}$ came from both intra- and extracellular sources. Through the use of the Ca^{2+} chelator EGTA it was demonstrated that the major source of $_{i}Ca^{2+}$ was extracellular with some contribution from ER stores. In addition to Ca^{2+} we also investigated inhibition of fsk-induced cAMP formation as a readout for S1P1 receptor signalling. The data confirmed persistent signalling of internalised S1P receptors from the TGN in astrocytes. The result also showed that MNP301 pre-treatment while preventing internalisation of S1P1 receptors to the TGN in addition to inhibition of Ca^{2+} signalling had no affect on S1P1 receptor persistent signalling to cAMP. Taken together the data suggests that MNP301 in addition to its inhibitory effect on FTY720-P induced internalisation is a selective antagonist for S1P1 receptor evoked $_{i}Ca^{2+}$ signalling without affecting the cAMP signalling pathway.

4.3.1 AUY954 induced activation of S1P1 receptors evokes oscillatory $_{i}Ca^{2+}$ waves.

It has been shown that Ca^{2+} signals may appear as a single transient elevation in $_{i}Ca^{2+}$, or as repeated Ca^{2+} transients, known as Ca^{2+} oscillations (Uhlen and Fritz, 2010). It was observed that AUY954 induced a noticeably different Ca^{2+} response to that of S1P. Specifically AUY954 treatment evoked an oscillatory calcium signalling event in contrast to S1P treatment which induced a transient increase in $_{i}Ca^{2+}$ followed by a relatively fast return to basal Ca^{2+} levels (**Fig.** 4.2). This differential Ca^{2+} response may be due to AUY954's selectivity for S1P1 receptors resulting in oscillatory responses which can be counteracted by involvement of S1P3 and S1P5 receptor evoked Ca^{2+} signals induced by S1P. The simplest model of Ca^{2+} oscillations is based upon the positive and negative modulatory effects of Ca^{2+} on IP3 receptor channels. When $_{i}Ca^{2+}$ levels fall below a certain threshold (~300nM), Ca^{2+} potentiates an IP3 receptor triggered release of Ca^{2+} (Bezprozvanny et al., 1991). This results in Ca^{2+} induced Ca^{2+}release (positive feedback) (Goldbeter et al, 1990). A rise above these levels results in negative feedback with Ca^{2+} being pumped into intracellular stores (Bezprozvanny et al., 1991). The ER Ca^{2+} levels are then restored back to resting levels by the ER bound SERCA pumps and SOCE (Uhlen and Fritz, 2010). Ca^{2+} oscillations only occur when positive feedback is faster than negative (Sneyd *et al.*, 1995; Politi *et al.*, 2006). Oscillatory Ca^{2+} signalling are well documented for G_q coupled GPCRs (De Bock et al., 2012). This is interesting as S1P1 receptors are exclusively G_i coupled, which implies that AUY954 stimulates Ca^{2+} release via the PLC/IP3 pathway through the βγ subunits of the G_i protein (Yoon et al., 2008).

4.3.2 S1P receptor internalisation states alter S1P receptors ability to induce Ca^{2+} signalling.

We confirm that S1P receptors can persistently inhibit fsk-induced cAMP formation from intracellular compartments in primary rat astrocytes (**Fig.** 4.10). This is thought to occur due to conformational changes in FTY720-P bound S1P receptors that 'lock' the receptor in an activated state, and/or prevent modifications that would lead to the receptor being degraded or recycled. It is hypothesised that FTY720-P bound S1P receptors are trafficked to intracellular compartments coupled to elements of the AC/cAMP signalling pathway. Here we showed that FTY720-P bound S1P receptors do not persistently signal in a Ca^{2+} dependent manner from intracellular compartments (**Fig.** 4.4). The data indicate that S1P receptors must be associated with the plasma membrane to evoke Ca^{2+} but not cAMP signalling events. Through the use of the S1P1 receptor specific compound AUY954, we demonstrate that S1P1 receptors contribute to ~50% of S1P induced Ca^{2+} signalling in primary rat astrocytes (**Fig.** 4.5). This result can be explained by the fact that S1P evokes Ca^{2+} signalling via both S1P1 and S1P3 receptors. Following astrocyte pre-treatment with AUY954, the

resulting stimulation with S1P led to a 50% reduction in Ca^{2+} responses (**Fig.** 4.5). In addition stimulation with AUY954 following pre-treatment with the same compound lead to a complete inhibition of Ca^{2+} signalling events (**Fig.** 4.5).

4.3.3 S1P1 receptor activates both intracellular Ca^{2+} release and extracellular Ca^{2+} influx.

Ca^{2+} signalling in human astrocytes was induced through activation of S1P1 receptors, by the S1P1 specific compound SEW2871. This signalling was slow to develop; lacking the transient peak observed in AUY954 induced Ca^{2+} responses in rat astrocytes. The different Ca^{2+} responses associated in SEW2871 and AUY954 are likely due to the different efficacies of these compounds in activating S1P1 receptors. Dantrolene inhibits Ca^{2+} release from intracellular stores through its actions on both IP3 receptors and ryanodine receptors. Cells were pre-treated with this compound ($\pm Ca^{2+}$ in the buffer solution) and then activated by the S1P1 receptor specific agonist SEW2871. A reduced initial Ca^{2+} peak was observed compared to control both in the presence and absence of extracellular Ca^{2+} (**Fig.** 4.9). This was exacerbated when Ca^{2+} was absent in the extracellular environment. However both in the presence and absence of extracellular Ca^{2+} a secondary increase in $_{i}Ca^{2+}$ levels was observed. These results suggest that activation of IP3 receptors on intracellular stores contribute to initial increase in Ca^{2+} in the cytosol. Ca^{2+} signalling after this point however, may be due to other stores of calcium, or most likely extracellular sources. Interestingly, $_{i}Ca^{2+}$ levels in cells pre-treated with dantrolene ($-Ca^{2+}$ in buffer solution), began to increase approximately 60secs after stimulation by SEW2871. Theoretically, there should not have been any Ca^{2+} available for signalling with ER stores blocked by dantrolene and extracellular Ca^{2+} removed ($-Ca^{2+}$ in buffer solution), nevertheless, an increase in $_{i}Ca^{2+}$ was observed. This response may have been due to an incomplete blockage of IP3 receptors by dantrolene or activation of other intracellular calcium source such as mitochondria. Cells were pre-treated with the irreversible calcium chelator EGTA ($-Ca^{2+}$) to determine the contribution of extracellular Ca^{2+} in S1P1 receptor evoked increases in $_{i}Ca^{2+}$ levels. Cells that underwent this pre-treatment did not respond to activation by SEW2871 (**Fig.** 4.9), either initially or with a delayed increase in $_{i}Ca^{2+}$. On the other hand, cells pre-treated with dantrolene in the presence of extracellular Ca^{2+} maintained their delayed elevation in $_{i}Ca^{2+}$. These data suggest that the delayed, sustained elevation in cytosolic Ca^{2+} is due to influx of extracellular Ca^{2+}. This model of astrocytic Ca^{2+} signalling is consistent with SOCE whereby S1P1 receptor induced activation of the PLC/IP3 pathway results in a transient spike in $_{i}Ca^{2+}$ levels, which in turn stimulates the slow sustained movement of Ca^{2+} across the plasma membrane. Interestingly, in cells pre-treated with EGTA ($-Ca^{2+}$ in buffer solution), basal $_{i}Ca^{2+}$ levels steadily increased throughout the

imaging period. This is possibly due to slow leak of Ca^{2+} from intracellular stores such as the ER or mitochondria that would not have been affected by EGTA treatment. This experiment does not fully delineate the source of $_{i}Ca^{2+}$ in astrocytes stimulated with S1P receptor agonists; however it does suggest involvement of both intra- and extracellular Ca^{2+} in S1P1 receptor induced increases in $_{i}Ca^{2+}$ levels, with extracellular Ca^{2+} playing a more prominent role.

4.3.4 MNP301: A pathway specific inhibitor of S1P1 receptor signalling.

Data indicate that MNP301 preferentially antagonises S1P receptor evoked Ca^{2+} signalling but not signalling to the AC/cAMP pathway. We hypothesise as to the molecular basis of this intriguing property of MNP301 (**Fig.** 4.12). MNP301 is designed to match the final 10 amino acids of the c-terminus of the S1P1 receptor. We hypothesise that the physiological effect of MNP301 is due to interference with protein-protein interactions at the c-terminus of the receptor, most likely via competitive inhibition of interacting proteins with S1P1 receptor binding pockets. It is thought that agonist binding alters the conformation of the 1st, 2nd and 3rd intracellular loops, thus uncovering previously hidden G-protein interacting sites (Hanson et al., 2012). In agreement with this MNP301 does not alter the G_i driven inhibition of fsk-induced cAMP formation. Similarly, the c-terminus has been shown to be crucial for agonist induced internalisation of the S1P1 receptor, which is in the agreement with the notion that MNP301 inhibits FTY270-P induced internalisation of the receptor to the TGN via a specific effect on interactions at the c-terminus. Finally, it is not understood how G_i coupled S1P1 receptors mediate Ca^{2+} signalling. While some studies suggest a role for the βγ subunits of the G_i protein, our findings indicate an additional involvement of the c-terminus (Birnbaumer, 1992). We hypothesise that a secondary complex is involved in coupling S1P1 receptors to the PLC pathway. This may or may not be the same complex associated with FTY720-P induced receptor internalisation. Identification of the proteins involved in this complex represents a key aim for future work in this field.

4.3.5 Uncoupling persistent signalling from persistent internalisation.

The data show that despite attenuation of S1P1 receptor internalisation by MNP301 (**Fig.** 3.10), the persistent signalling (persistent cAMP inhibition) caused by FTY720-P treatment was not altered by pre-treatment with MNP301 (**Fig.** 4.10, lane 4). Collectively, we conclude that while MNP301 prevents FTY720-induced internalisation of S1P1 receptors to the TGN, this peptide does not affect G_i protein mediated signalling of these receptors.

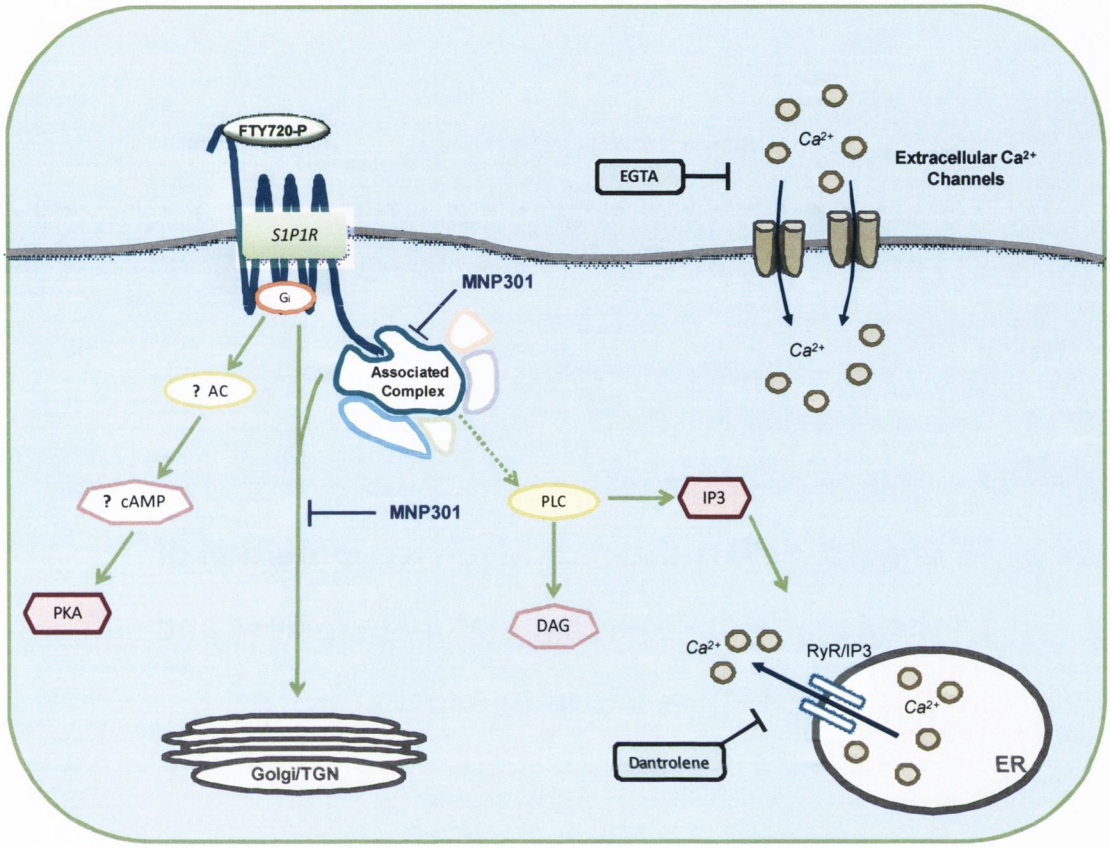


Figure 4.12 *MNP301: A pathway specific inhibitor of S1P1 receptor signalling.*

We have shown that the S1P1 specific blocking peptide MNP301 inhibits FTY720-P induced internalisation of the S1P1 receptor to the TGN. In addition MNP301 selectively inhibits S1P1 mediated increases in $_{i}Ca^{2+}$ levels in primary rat astrocytes (**Fig.** 4.6) while having no effect on G_i mediated inhibition of fsk-induced cAMP production (**Fig.** 4.10). This pathway specific effect on one downstream signalling pathway versus another is intriguing both pharmacologically and physiologically. We hypothesise that MNP301 alters both internalisation and Ca^{2+} signalling via competitive inhibition of an interaction between the extreme c-terminus of the S1P1 receptor and an unknown associated complex. This complex may couple the G_i coupled GPCR to the PLC/IP3 pathway, and may be the same or similar to the molecular machinery needed for internalisation of FTY720-P bound S1P1 receptor to the TGN.

Chapter 5. S1P Receptor Mediated Inhibition of the Release of Pro-Inflammatory Cytokines and Chemokines from Astrocytes.

Aims

1: To identify the range of chemokines/cytokines released from primary rat astrocytes in response to an LPS challenge.

2: To investigate the effect pre-treatment with the S1P1, 3 and 5 receptor agonist FTY720-P has on LPS stimulated primary rat astrocytes.

3: To determine a role for S1P1 receptors in the inhibition of LIX release from primary rat astrocytes, using the S1P1 receptor specific compound SEW2871.

4: To examine the effect of S1P1 receptor activation on receptor internalisation in human astrocytes.

5: To study the effect of S1P on pro-inflammatory cytokine release from human astrocytes.

6: To study the effect of IL-17 and TNF-α on pro-inflammatory cytokine release from human astrocytes.

7: To investigate the effect of synergistic signalling between IL-17 and TNF-α on IL-6 release from human astrocytes.

8: To investigate the specific role of S1P1 receptors in the inhibition of pro-inflammatory cytokine induced IL-6 release from human astrocytes

9: To investigate the role of SphK1 in IL-17/TNF-α induced IL-6 release from human astrocytes.

Abstract

S1P receptors are a family of classical G-protein coupled receptors and are composed of five subtypes S1P1-S1P5. These receptors are the molecular targets of Gilenya® (Fingolimod; FTY720), the first approved oral therapy for MS. To further examine the function of S1P1 receptors in the CNS, their role in cytokine release from both rat and human astrocytes was demonstrated. Here, focus was given to an IL-17 induced increase in levels of IL-6, both of which have been implicated as pro-inflammatory cytokines in MS. The data showed that human astrocytes expressed IL-17 receptors and *in vitro* treatment of human astrocytes with IL-17 increased the levels of IL-6, which was greatly enhanced in the presence TNF-α, above and beyond an additive effect of the two cytokines. Importantly, the treatment of human astrocytes with S1P also increased the levels of IL-6. In contrast, FTY720-P inhibited the IL-17/TNF-α induced increase in levels of IL-6, as did the S1P1 receptor selective compound AUY954. Notably, both FTY720-P and AUY954, but not S1P, internalised S1P1 receptors which likely explains the opposing effects of these S1P receptor ligands on regulating the levels of IL-6. Of interest the effects of IL-17 were not associated with any involvement of the sphingosine kinase pathway. In addition to regulating IL-17 induced IL-6 release, both FTY720-P and AUY954 inhibited the release of a number of chemokines from LPS stimulated rat astrocytes. Taken together, the data suggested that FTY720-P attenuates the release of pro-inflammatory cytokines and chemokines from both rat and human astrocytes stimulated with either a combination of IL-17 and TNF-α or the bacterial endotoxin LPS. This inhibition of cytokine/chemokine release appears to be mediated via the S1P1 receptor. The FTY720-P mediated shut down of IL-17/IL-6 signals in astrocytes likely contributes to the ability of this drug to reduce the pro-neuroinflammatory signals that sustain MS.

5.1 Introduction

Following our findings that FTY720-P can modulate S1P receptors in astrocytes, we investigated further their role in this cell type. The CNS has relatively low regenerative capabilities, due in part to the post-mitotic status of its neurons, thus under normal conditions the access of immune cells to the CNS is tightly regulated. However, disruption of the BBB and focal infiltration of autoreactive leukocytes into the CNS compartment are hallmarks of MS (Palmer, 2010). We believe that astrocytic cross-talk with cells of the innate immune cells, in particular Th1 and Th17 cells results in astrocytes driving neuroinflammation through the release of chemokines and subsequent recruitment of leukocytes into the CNS (Chastain and Miller, 2012). Antigen experienced T-cells are crucial components of the body's immune system and are vital in the rapid production of a robust immune response to foreign pathogens (Kumar et al., 2011). However many autoimmune disorders occur as a direct result of reactivation of autoreactive memory T-cells which recognize "self-antigens" and in the case of MS, antigens specific to the CNS. In this section we introduce the key roles several immune cells and their secreted cytokines play in the pathogenesis of MS. In this chapter the focus shifts from the trafficking and signalling of the S1P1 receptor to examining the role astrocytes play as active mediators of neuroinflammation via the production and release of various pro-inflammatory cytokines and chemokines. In addition to the effect of S1P receptor modulation on the release of these inflammatory mediators was examined.

5.1.1 T-cells: Classification and circulation.

Antigen specific memory T-cells are a pool of antigen experienced lymphocytes that are acquired over the lifetime of an individual. This pool of cells can be divided into two sub-populations, namely 'central memory T-cells' (T_{CM}) and 'effector memory T-cells' (T_{EM}) (Sallusto et al., 2004) (**Fig.** 5.1). These populations can be distinguished by the differential expression of the chemokine receptor CCR7 and the cell adhesion molecule L-selectin (CD62L). Both subsets can be $CD4^+$ or $CD8^+$ or a combination of both and typically express the cell surface protein CD45RO. T_{EM}'s are defined by the cell surface expression profile $CCR7^-$, $CD3^+$, $CD45^-$, L-$selectin^{Low}$ (Obar and Lefrancois, 2010). These cells gain entry to non-lymphoid tissues where upon re-stimulation through positive interactions with professional APC's they play a major role in eliciting an inflammatory response (Almolda et al., 2011). T_{EM} do not participate in the process of lymphoid recirculation as observed in the T_{CM} subtype (Mehling et al., 2008). This is due to their low expression of CD62L and CCR7 (Chiba et al., 2005). T-cells enter lymph nodes following a process of 'rolling', CD62L mediated interaction with the adhesion molecule, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and CD34

molecules found on the surface of high endothelial venules which causes weak binding of the T-cell to the endothelial cells (Chiba, 2005). CCL21 and possibly CCL19 is released by the high endothelial venules, these chemokines signal to the T-cell via the chemokine receptor CCR7 (Henning et al. 2001). This signalling causes a conformational change in the cells LFA-1 molecule initiating a tighter binding with the intercellular adhesion molecule, (ICAM-1) found on the surface of the endothelial cells (Warnock et al., 1998). This whole process stabilizes the movement of the T-cell and allows the cell to cross through the high endothelial venules into the lymph nodes in a process termed "diapedesis" (Warnock et al., 1998).

Unlike the T_{EM} subtype which have irreversibly lost their expression of CCR7, T_{CM}'s are $CCR7^{+}$, which brings this sub-population under the influence of the CCL21/CCL19 retention signals derived from the endothelial cells of the lymph nodes (Parish and Kaech, 2009). Naive T-cells (T_0) and T_{CM}'s recirculate between the peripheral blood and secondary lymphoid organs and home to lymph nodes (Warnock et al., 1998). Here these cells respond to CCR7 mediated retention signals. To override this signal and egress from the lymph nodes these cells require activation of S1P1 receptors found on their cell surface (Matloubian et al., 2004). This occurs through the autocrine actions of lymphatic endothelial cell derived S1P or possibly from an S1P gradient stemming from the CNS. Whatever the source, S1P allows the cells to overcome the CCR7 mediated retention signal and results in the egress of T_0 and T_{CM} from the lymph nodes (Henning et al., 2001). The conflicting S1P-S1P1 receptor egress and CCL19/21-CCR7 retention signals has been shown to influence both the circulation of mature T-cells from bone marrow and the regulation of systemic T-cell circulation (Maeda et al., 2010; Pham et al., 2008). The cells respond to the more dominant signal, with many factors influencing the establishment of a signal. While the molecular basis of this phenomenon is not fully understood, it is thought to involve the dominant signalling receptor competing for the cytoskeletal machinery required to drive polarity and cellular mobility (Lo et al., 2005).

FTY720-P binds to and internalises S1P1 receptors found on T-cells, impairing their ability to respond to the aforementioned S1P signal (Pham et al. 2008). This down-modulation of T_0 and T_{CM} S1P1 receptors by FTY720-P results in the CCR7 dependent retention of these cells in the lymph nodes. This is turn results in a large reduction in the number of T_0 and T_{CM} cells found in the blood of FTY720-P treated MS patients, with the number of T_{EM}'s remaining unaffected (Mehling et al. 2008; Metzler et al. 2008; Brinkmann et al. 2009). As such this set of molecular events culminating in the inhibition of S1P-mediated egress of T-cells from the lymphoid tissues represents the main mode of action of Gilenya® (Mehling et al., 2010). While the data suggests that T_{CM} are the primary target of

FTY720-P it remains highly unlikely given the widespread expression of the S1P receptors coupled with the lipophilicity of the drug that this is not the only interaction contributing to Gilenya's therapeutic effect. This study investigates the FTY720-P-S1P1 receptor interaction on astrocytes in the hope of discovering a disease relevant physiological effect of the internalisation and signalling events observed in the preceding chapter. Recent studies have provided compelling data suggesting that astrocytes are key drivers of neuroinflammation by providing chemotactic signals for infiltrating leukocytes which are essential for the progression of EAE (Kang et al., 2010). Thus, this chapter focuses on the ability of FTY720-P to prevent the release of pro-inflammatory chemokines and cytokines from activated astrocytes.

5.1.2 T-cells: Th1 versus Th2.

Classically in the field of autoimmunity and autoimmune diseases it was believed that a diseased state was caused by an imbalance in the Th1-Th2 paradigm. A Th1 response mediates a cellular immune response, associated with tissue damaging inflammation. In contrast, a Th2 response is characterised by shifting the overall immune response away from a cell-mediated to a humoral based response exerting a modulatory or anti-inflammatory effect (Li et al., 2011). However this concept that MS is a purely Th1 mediated disease was first challenged by the several findings and observations in the animal model EAE (Ferber et al., 1996).

It was observed that IFN-$\gamma^{-/-}$ or STAT1$^{-/-}$ mice which do not generate Th1 cells were not only susceptible to EAE but found to develop a more severe form of EAE (Bettelli et al., 2004; Ferber et al., 1996). Importantly it has been shown that the pro-inflammatory cytokine IL-12 promotes the development of IFN-γ producing, Th1 cells (Watford et al., 2003). IL-12 shares the p40 subunit with another cytokine, namely, IL-23. It was observed that IL-12p40$^{-/-}$ mice were resistant to EAE, subsequently it was shown that IL-23$^{-/-}$ were also resistant to the development of both EAE and collagen induced arthritis, a well established animal model for the autoimmune disease, rheumatoid arthritis (Cua et al., 2003; Murphy et al., 2003). IL-23 is a member of the IL-12 cytokine family; the main cellular sources of IL-23 are professional APCs, activated macrophages and dendritic cells (Cua et al., 2003). The main function of IL-23 in conjunction with IL-6 and TGF-β1 is to enhance the expansion of IL-17, IL-17F, and IL-22 producing T-cells and to maintain this population of pro-inflammatory cells (Langrish et al., 2005). It is thought that while IL-23 is not necessary for the initial differentiation of Th17 cells from naive T-cells, it is required for the terminal differentiation and thereby ultimately the cells activity. Recent studies have described an important role for CD4^{+} Th17 cells in the effector stage of EAE mediated through the production of pro-inflammatory IL-17. It is

now known that Th17 and Th1 cells can independently induce phenotypically distinct EAE via separate immunological pathways, with respect to both histology and chemokine expression profiles (Kroenke et al., 2008).

5.1.3 Pro-inflammatory cytokine: IL-17.

IL-17 is the founding member of the IL-17 family of cytokines (IL-17 A-F). It is a pro-inflammatory molecule and the signature cytokine of the Th17 cell subclass. It was first discovered and cloned by Rouvier et al and named CTLA8, it has since been re-named IL-17 (IL-17A) (Rouvier et al., 1993). IL-17 is a 155 amino acid long homodimer which exerts its effect through binding to and activation of a type-I cell surface receptor. IL-17RA and IL-RC are the best described of the 5-known IL-17R's and respond to both IL-17A and IL-17F (Yao et al., 1995; Zheng et al., 2008). While Th17 cells are the main source of IL-17 they are not the only cells capable of producing this cytokine. Natural killer cells, γδ T-cells and $CD8^+$ T-cells have all been shown to be capable of producing IL-17 under various conditions (Lockhart et al., 2006; Rachitskaya et al., 2008; Sutton et al., 2006). The role of IL-17 signalling has been investigated in a number of inflammatory disease states including psoriasis, inflammatory bowel disease, and rheumatoid arthritis; however we are most interested in its role in EAE/MS (Jadidi-Niaragh and Mirshafiey, 2011). Studies show anti-IL-17 neutralising antibodies can both delay the onset and reverse the progression of established EAE, in addition, $IL\text{-}17^{-/-}$ mice show a reduced susceptibility to EAE (Hofstetter et al., 2005). While this genetic or biological interference with the IL-17 signalling pathway appears to be beneficial in EAE, it only leads to amelioration of clinical symptoms not prevention or complete reduction of the symptoms (Hofstetter et al., 2005; Park et al., 2005).

Various studies have examined the role of Th17 cells and IL-17 in autoimmune disease. It has been shown that there is a significant increase in IL-17 at an mRNA and a protein level in both active and chronic MS lesions as compared to control or normal appearing white matter (Traugott et al., 1983; Tzartos et al., 2008). It has also been shown that there is increased numbers of mononuclear cells expressing IL-17 mRNA in both the blood and CSF of patients with active MS (Matusevicius et al., 1999). Increases in these cell numbers also correlated to periods of clinical exacerbation such as in a disease relapse (Durelli et al., 2009; Matusevicius et al., 1999). The main pathogenic function of IL-17 involves the recruitment of both neutrophils and macrophages during inflammation (Witowski et al., 2004). Subsequently neutrophils mediate the activation of various gelatinases and proteinases, including matrix metalloproteinase-3 (MMP-3), which actively disrupt the BBB (Yong et al., 2001). This BBB breakdown is facilitated by IL-17 signalling through the IL-17R and enhances the production

of reactive oxygen species (ROS) in CNS endothelial cells (Huppert et al., 2010). Consequently this leads to disorganisation or loss of tight junction proteins in these endothelial cells and permeabilization of the BBB (Huppert et al., 2010). Altogether these studies suggest that IL-17 or IL-17R's represent targets for future immunotherapeutics. However the situation is complicated somewhat by the fact that Th17 cells not only produce IL-17 but also a range of bioactive molecules including IL-6, TNF-α, IL-21 and IL-22. Thus, further studies on the role of Th17 cells and IL-17 in the inflammatory process are needed before IL-17/IL-17R's become a legitimate druggable target for the treatment of autoimmune diseases. However, from cytokine knockout studies done in mice, it has been demonstrated that no one cytokine has been identified as the key inflammatory mediator in the course EAE (Ferber et al., 1996; Liu et al., 1998).

5.1.4 Pro-inflammatory cytokine: IL-6.

One of the main biological targets of IL-17 signalling is the pleiotropic cytokine IL-6, first described in 1986 (Haegeman et al., 1986). IL-6 plays various roles in the human body; of particular interest is its critical role in the inflammatory process (Neurath and Finotto, 2011). IL-6 is a potent mediator of fever and along with IL-8, IL-1 and TNF-α is a stimulator of the acute-phase reaction (Neurath and Finotto, 2011). IL-6 can be secreted by many cells including monocytes, endothelial cells, T-cells, and B-cells with astrocytes thought to be the primary source of IL-6 within the CNS (Van Wagoner et al., 1999). In the healthy CNS only very low levels of IL-6 can be detected, however this is markedly increased after injury (Yan et al., 1992). IL-6 release in the CNS has been shown to exert both beneficial and detrimental effects. IL-6 plays an important role as a trophic factor within the CNS, protecting and promoting the survival of both undifferentiated and mature neurons (Gruol and Nelson, 1997). IL-6 also has been shown to promote the release of NGF from cultured astrocytes (Frei et al., 1989). Given the pleiotropic nature of IL-6, its transient production can have a positive effect on host defenses. Chronically dysregulated IL-6 levels are implicated in many inflammatory and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and MS (Murakami and Nishimoto, 2011; Ogata and Tanaka, 2012). IL-6R blockade has already been targeted in the treatment of RA with the monoclonal antibody Tocilizumab® (Hoffmann-La Roche) (Ogata and Tanaka, 2012) IL-6 crosses the BBB easily and at pathological levels is thought to promote a pro-inflammatory milieu (Banks et al., 1995). Numerous studies have shown elevated levels of IL-6 in the serum and CSF of patients suffering from conditions such as, both viral and bacterial meningitis, MS, Alzheimer's disease and systemic lupus erthematosus (Gruol and Nelson, 1997). In the context of EAE and MS, studies have shown that IL-6 mediates many of its pro-inflammatory effect via its ability

to promote Th17 cell differentiation, while inhibiting the expansion of the anti-inflammatory Treg population (Kimura and Kishimoto, 2010). Dysregulation of IL-6 and IL-6 signalling pathways has long been implicated in various autoimmune diseases and in particular rheumatoid arthritis (Murakami and Nishimoto, 2011). It is this evidence of IL-6 mediated imbalance in Th17/Treg populations that has lead IL-6 to become a target in the search for new anti-autoimmune therapies.

5.1.5 Pro-inflammatory cytokine: TNF-α.

Similar to IL-6, TNF-α (first described in 1975) is a cytokine with diverse cellular functions (Carswell et al., 1975). It is best known for its involvement in systemic inflammation and as a stimulator of the acute phase reaction (Locksley et al., 2001). TNF-α signals through two membrane bound receptors (TNF-R1 and TNF-R2), it is a strong activator of the NF-$_{\kappa}$B and MAP kinase signalling pathways, in particular the JNK (c-Jun N-terminal kinase) pathway (Davis, 1999). Macrophages are the main cellular source of TNF-α, however various other cell types are capable of producing TNF-α including microglia, astrocytes, mast cells and endothelial cells. Elevated serum and CSF levels of TNF-α have been observed in patients suffering from MS (Martins et al., 2011; Sharief and Hentges, 1991; Shi et al., 2009). The pro-inflammatory effects of TNF-α in the acute phase of EAE has also been well documented, however attempts to target TNF-α/TNF-R signalling as a treatment strategy for MS have been unsuccessful (Group, 1999).

5.1.6 Chemotactic cytokines.

Chemokines are a superfamily of small cytokines; these chemotactic cytokines are secreted by various cells of the body and are involved in the recruiting, trafficking and redistribution of leukocytes (Kehrl, 2006). Chemokines are segregated according to differences in their structure, and in particular the position of the first two cysteine residue in these molecules. The two largest subfamilies of chemokines are the CC family (which has its first two cysteine residues next to each other) and the CXC family (which contain a single amino acid between its first two cysteine residues) (Charo and Ransohoff, 2006). The primary role of chemokines is to direct the movement of circulating leukocytes to sites of inflammation or injury (Kehrl, 2006). Chemokines signal through seven transmembrane GPCRs, to a number of downstream signalling pathways, including the PLC pathway (chemokine activation of leukocytes induces increased levels of $_{i}Ca^{2+}$) and the AC/cAMP pathway (chemokine induced decreases in cAMP levels enhances chemotaxis in leukocytes (Li et al., 2000). There are 19 known chemokine receptors; these receptors can be activated by a number of different chemokines, thus there exists a degree of redundancy in chemokine signalling. It is this

redundancy that makes chemokines and their receptors difficult drug targets (Xu et al., 2007). Maraviroc (CCR5 receptor antagonist), a compound used as a HIV therapy, is the only licensed therapy which targets chemokine receptors (Westby and van der Ryst, 2010).Multiple chemokine receptor antagonism has been suggested as a being the most effective approach in targeting the chemokine network (Zhao, 2010). What is clear is the role chemokine play in disease, in particular autoimmune diseases and disorders with a prominent inflammatory component, such as psoriasis, asthma and MS (Flier et al., 2001; Pease, 2011; Trebst et al., 2001). The release from astrocytes of two chemokines, one from the CC family (CCL5; RANTES) and one from the CXC family (CXCL5; LIX) was examined in this study.

5.1.7 Pro-inflammatory chemokine: RANTES.

RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) or CCL5 is a 8kDa chemotactic and pro-inflammatory cytokine of the CC subfamily. RANTES plays an active role in recruiting leukocytes to sites of inflammation, it has been shown to interact with and activate CCR3, CCR5 and CCR1 (Proudfoot et al., 2001; Slimani et al., 2003; Struyf et al., 2001). RANTES is chemotactic for T cells and eosinophils and induces proliferation and activation of a subset of NK cells, known as CC-chemokine-activated killer (CHAK) cells (Maghazachi et al., 1996). RANTES displays potent basophil chemotactic activity, HIV-suppressive activity (as produced by $CD8^+$ cells) and anti-microbial action in the innate immune response (Miller and Krangel, 1992). RANTES expression has been observed in astrocytes, in the MS brain, and in control cultures (Meeuwsen et al., 2003; Szczucinski and Losy, 2011). Expression of RANTES receptors CCR1 and CCR5 on astrocytes has also been demonstrated (Dorf et al., 2000). Release of RANTES from murine astrocytes has been successfully evoked *in vitro*(van Neerven et al., 2010). Even in the nanomolar range, RANTES, eotaxin and MIP-1α/β induced expression of monocyte chemoattractant protein-1 (MCP-1), TNF-α, MIP-1α, MIP-2 and RANTES, in a pertussis toxin-sensitive manner, and via multiple receptors, including CCR1 and CCR5 (Luo et al., 2002). The ability of astrocytes to respond to, and produce RANTES is noteworthy, as autocrine and paracrine methods of RANTES signalling within astrocyte populations may serve to activate astrocytes and amplify/prolong inflammatory responses within the CNS. Downstream effects of RANTES signalling invokes phosphorylation of several key targets, including MEK, ERK1/2, p90 ribosomal S6 kinases (RSKs) and cyclic AMP-response element-binding protein (CREB) in astrocytes (Zhang et al., 2002). Inhibition of this pathway via U0126, a pharmacological inhibitor of MEK, attenuates further downstream phosphorylation events, as well and RANTES-induced chemokine synthesis (Zhang et al., 2002). RANTES signalling has also been demonstrated to

modulate astrocyte receptor expression; in particular, ICAM-1 is upregulated, and CX3CR1 expression down regulated following treatment of astrocytes with RANTES (Luo et al., 2002). It has been reported that RANTES plays an important role during human foetal astrocyte development, whereby RANTES differentially induced proliferation without differentiation, or differentiation without proliferation, depending on the foetal age (5 weeks versus 10 weeks, respectively) (Bakhiet et al., 2001).

5.1.8 Pro-inflammatory chemokine: LIX.

It is postulated that astrocytes are the significant CNS source of granulocyte-specific chemokines (Lu et al., 2005). LIX (lipopolysaccharide induced CXC chemokine) or CXCL5 is a small cytokine belonging to the CXC chemokine family first cloned in 1994 (Chang et al., 1994). It was characterised as an inducible factor following stimulation of cells with the inflammatory cytokines IL-1 and TNF-α (Chang et al., 1994). Its principal role seems to be the promotion of chemotaxis of neutrophils specifically possessing angiogenic properties. LIX elicits its effects through interaction with the CXCR2 chemokine receptor (Persson et al., 2003). The chemokine CXCL3 which is implicated in the control of migration and adhesion of monocytes in the periphery also signals through the CXCR2 receptor (Lu et al., 2005). The combined action of these two chemokines on this receptor suggests that the receptor itself may play an important role in MS pathology, and that efficacy of FTY720-P may be contributed to by a down-regulation of pro-inflammatory factors such as LIX. CXCR2 has been previously implicated in disorders of inflammation, myelin and oligodendroglial biology (Charo and Ransohoff, 2006). A recent study on the cuprizone induced demyelination model of MS revealed that $CXCR2^{-/-}$ mice are relatively resistant to cuprizone induced demyelination, and that $CXCR2^{+}$ neutrophils are essential for cuprizone-induced demyelination (Liu et al., 2010).

5.1.9 Chapter Aim.

A recent study showed that a combination treatment of both IL-17 and TNF-α *in vitro* caused an increase in oligodendrocyte cell loss compared to treatment with the cytokines individually (Paintlia et al., 2011). This synergistic effect was above and beyond any possible additive effect of using two cytokines. This exacerbation of IL-17 induced oligodendrocyte apoptosis by TNF-α was characterised by mitochondrial dysfunction, generation of ROS and the induction of cell cycle arrest (Paintlia et al., 2011). Synergistic activity is intriguing in terms of inflammation in EAE/MS, and indicates possible interplay between IL-17 producing Th17 cells and TNF-α producing Th1 cells. The overall aim of this chapter was to further understand the role astrocytes play in an inflammatory environment. In an

EAE or MS setting, chemotactic molecules attract tissue damaging leukocytes to sites of inflammation in the CNS. LPS is the most frequently utilised *in vitro* model stimulus for inflammation (Lund et al., 2006). This was an adequate approach to first study the effects of inflammatory signals in astrocytes, and also to determine the effects of FTY720-P on this activation. To further understand the role astrocytes might play in an autoimmune setting such as MS, follow-up studies in this chapter utilised more "disease-relevant" molecules to treat astrocytes. Specifically, human recombinant IL-17 and TNF-α were used to stimulate astrocytes, both in parallel and in conjunction with one another. IL-6 release was subsequently measured as an inflammatory read-out from these cells. The effect of FTY720-P on IL-17 and TNF-α induced release of IL-6 was then demonstrated.

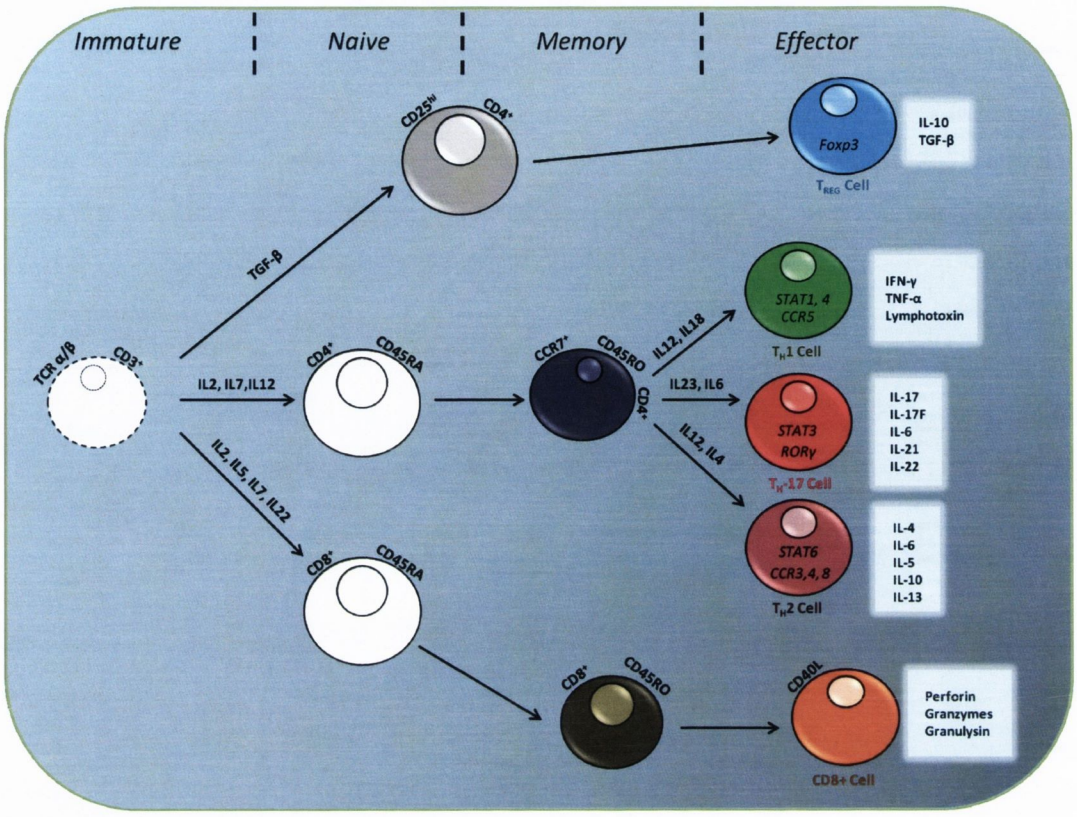


Figure 5.1 *T-Cell lineages.*

T-cells go through various stages of development before becoming distinct effector cells. From left to right, non-renewing hematopoietic stem cell progenitors migrate from the bone marrow to the thymus where they undergo further development, slowly losing their ability to develop down different cellular lineages. Through the processes of positive and negative selection the thymus eventually produces and releases single-positive ($CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$) immunocompetent naive thymocytes. After activation of the naive T-cell through its TCR and co-stimulatory molecules by APCs, the T-cells re-circulate to the lymphoid organs to develop into a population of antigen experienced memory T-cells which await reactivation. These cells given the right cytokine environment (as indicated) can become any one of the distinct effector T-cells which are capable of affecting the immune response through the production of a range of biologically active cytokines and chemokines.

5.2.3 LPS induces the release of a range of cytokines and chemokines from rat astrocytes.

We are interested in the role S1P receptor activation plays during inflammation; studies have demonstrated cooperation between TLR4 receptors and S1P1 and S1P3 receptors in the production of cytokines and chemokines (Eskan et al., 2008). Recently it has also been shown that inhibition of SphK1 can prevent sepsis in mice treated with LPS (Puneet et al., 2010). LPS is a well established cellular challenge which is known to elicit a strong inflammatory phenotype in many cell types including astrocytes (Bal-Price and Brown, 2001). LPS is a lipoglycan found on the outer membrane of Gram-negative bacteria which acts as an endotoxin and signals through the single membrane spanning CD14/TLR4/MD2 receptor complex (**Fig.** 5.2). This induces the production and secretion of various pro-inflammatory cytokines from many cell types (Rossol et al., 2011). TLR4 along with TLR3 has shown to be expressed on cells of the CNS, including astrocytes (Farina et al., 2007). In the present chapter to study the effect of S1P receptor modulation in an *in vitro* model of inflammation an "LPS challenge" was utilised. Primary rat astrocytes were exposed to 100ng/ml LPS for 3hr and 24hr to assess the effect of both a short and long LPS challenge on cytokine and chemokine release from astrocytes. Following stimulation supernatants were collected and analysed for the release of cytokines and chemokines using dots blot array kits. As shown in (**Fig.** 5.3) LPS induces astrocytes to release a wide range of pro-inflammatory cytokine and chemokines; CINC-2, CINC-3, IL1α, IL-6, IL-13, MIG, RANTES and TNF-alpha (*** $p<0.001$, one-way ANOVA, as determined by unpaired t-test **Fig** 5.3A), CINC-1, CNTF, GM-CSF, IP-10, L-Selectin, MIP-1β (** $p<0.001$, one-way ANOVA, as determined by unpaired t-test **Fig** 5.3A), IL-10 (* $p<0.001$, one-way ANOVA, as determined by unpaired t-test **Fig** 5.3A). Astrocyte stimulation with LPS for 24hr resulted in a similar cytokine/chemokine release pattern with a notable increase in IL-6 release. Data is represented as a fold increase in release of cytokines and chemokines following LPS treatment compared to control levels. This data confirms the release of a wide range of cytokines and chemokines from primary rat astrocytes in response to LPS activation of the TLR4 receptor. Following this initial experiment the chemokines RANTES (CCL5) and LIX (CXCL5) were selected for further investigation. These particular molecules provide a chemotactic signal for granulocytes.

5.2.4 FTY720-P pre-treatment attenuates LPS induced RANTES and LIX release from rat astrocytes.

To further assess possible S1P receptor mediated astrocytic involvement in the recruitment of immune cells to the CNS, the effect of FTY720-P treatment on the release of RANTES and LIX from

primary rat astrocytes in response to LPS stimulation was examined. Cells were starved in serum-free medium 4hr prior to a 2hr FTY720-P pre-incubation. Following pre-incubation, 100ng/ml LPS was added to the astrocytes for a 12hr treatment at 37°C/5% CO_2. The supernatants were collected and used on cytokine dot blots to determine release of cytokines and chemokines. A 100ng/ml concentration of LPS was sufficient to stimulate primary rat astrocytes to release significant amounts of both LIX (*** $p<0.001$ LPS vs. control; one-way ANOVA, Bonferroni post-hoc test) and RANTES (*** $p<0.001$ LPS vs. control; one-way ANOVA, Bonferroni post-hoc test). Pre-treatment with 1μM FTY720-P attenuated this LPS-induced release of LIX (### $p<0.001$ LPS + FTY720-P vs. LPS) and RANTES (### $p<0.001$ LPS + FTY720-P vs. LPS) *in vitro* (**Fig.** 5.4). Chemokine levels were observed to be returned close to control levels following FTY720-P pre-treatment. This result confirms that FTY720-P induced modulation of S1P receptors exerts anti-inflammatory effects on astrocytes.

5.2.5 S1P increases IL-6 levels in human astrocytes, in the presence of IL-17 and/or TNF-α.

S1P has been shown to promote the release of growth factors (NGF, FGF-2, and GDNF) from astrocytes that allow for cell-crosstalk and promote neuronal survival (Bassi et al., 2006; Yamagata et al., 2003). It has also been shown that FTY720-P reduces IL-6 levels in microglia/macrophages, astrocytes, and neurons following traumatic brain injury (Zhang et al., 2008). Additionally, IL-6 and IL-17 are increased during EAE and specific S1P1 receptor knockout from astrocytes or FTY720-P treatment reduces their release (Choi et al., 2011; Samoilova et al., 1998). Since previous studies show that foetal calf serum (FCS) contains between 126-156nM S1P, the effect of increasing concentrations of FCS on protein levels of IL-6 was first examined (Murata et al., 2000a). FCS induced an increase in the protein levels of IL-6 in conditioned media obtained from cultured human astrocytes in a concentration dependent manner (*** $p<0.001$ vs. corresponding control; one way ANOVA, Dunnetts post-hoc test) (**Fig.** 5.5A). Thus, all experiments conducted in this study were carried out in serum free conditions, where control levels of IL-6 were approximately 100pg/ml. Importantly, the treatment of astrocytes with S1P induced a concentration dependent increase in the protein levels of IL-6 in the astrocyte conditioned media (*** $p<0.001$ vs. corresponding control; one way ANOVA, Dunnetts post-hoc test) (**Fig.** 5.5B). In contrast to the ability of TNF-α to augment IL-17 mediated increase in protein levels of IL-6, the treatment of human astrocytes with IL-17 (**Fig.** 5.5C), TNF-α (**Fig.** 5.5D) or combined IL-17/TNF-α (**Fig.** 5.5E) did not enhance the S1P mediated increase in protein levels of IL-6, beyond the additive effects of these individual treatments (*** $p<0.001$ vs. corresponding control; one way ANOVA, Dunnetts post-hoc test). Thus, the data showed

that S1P increases the protein levels of IL-6 in human astrocytes and that the co-stimulation of TNF-α or IL-17 receptors did not alter this S1P response.

5.2.6 Synergistic effect of IL-17 and TNF-α signalling enhances the release of IL-6 from human astrocytes.

To investigate a possible role for astrocytes in propagating inflammatory responses, human astrocytes were treated with IL-17, the signature pro-inflammatory cytokine of Th17 cells. IL-17 is known to play a central role in neuroinflammation (Zepp et al., 2011). In parallel, astrocytes were treated with the established inflammatory molecule TNF-α, whose cognate receptor is known to be expressed on astrocytes and levels of which have been shown to be present at elevated levels in MS patients (Kinouchi et al., 1991; Martins et al., 2011). In addition, to determine the possible effect of synergistic signalling between IL-17 and TNF-α on IL-6 release from astrocytes, IL-6 protein levels were measured from astrocytes treated with a combination of both cytokines. Cells were starved in serum-free media for 4hr prior to stimulation with cytokines. Astrocytes were treated with increasing concentration of each cytokine for 18hr whereupon the media was removed and frozen at -20°C. IL-6 ELISA data indicates that treatment with IL-17 caused a significant increase in IL-6 from human astrocytes, with a maximal release of 1.3ng/ml of IL-6 following stimulation with 50ng/ml IL-17 (** $p<0.01$, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 5.6A). Similarly TNF-α treatment caused a significant increase in IL-6 release with a maximal release of approximately 2 ng/ml of IL-6 following stimulation with 10ng/ml TNF-α (** $p<0.01$, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 5.6B). Various combination of cytokines were used to treat human astrocytes, keeping the concentration of IL-17 constant while varying the TNF-α concentration and conversely maintaining the concentration of TNF-α constant while varying that of IL-17. Treatment of astrocytes with a combination of the cytokines (IL-17 50ng/ml and TNF-α 10ng/ml) resulted in the release of significantly higher concentrations of IL-6, with a maximal release of approximately 20 ng/ml IL-6 (** $p<0.01$, one-way ANOVA, Dunnetts post-hoc test; **Fig.** 5.6C). This effect was greater than the sum of the treatments with the individual cytokines alone (**Fig.** 5.6C). Here the data shows that astrocytes respond to pro-inflammatory signals and can contribute to the propagation of the inflammatory episode by releasing IL-6. The data also indicates synergistic signalling between IL-17 and TNF-α (Paintlia et al., 2011). This is relevant as it is more likely that in an inflammatory microenvironment astrocytes respond to a mixed cytokine environment and not one that is dominated by a single cytokine. This cooperative or synergistic signalling between inflammatory mediators is not a new concept (Chabaud et al., 2000). However, this profound increase in IL-6 release from human

astrocytes is of considerable interest, especially in the context of astrocytic involvement in driving neuroinflammation.

5.2.7 Pre-treatment of human astrocytes with FTY720-P causes a dose dependent inhibition of IL-6 release.

Previous data has shown that FTY720-P exerts an "anti-inflammatory" effect on LPS challenged astrocytes (**Fig.** 5.4). Data showed that pre-treatment of human astrocytes with FTY720-P attenuated the release of pro-inflammatory chemokines. Here our data suggests that Th17 cells may communicate with astrocytes to further drive inflammation through the promotion of IL-6 release (**Fig.** 5.6). Thus the possible further anti-inflammatory effect of FTY720-P on IL-17/TNF-α induced IL-6 release from human astrocytes was investigated. Human astrocytes were starved for 3hr in serum free media before they were treated with various concentrations of FTY720-P for 1hr. A combination treatment of 50ng/ml IL-17 and 10ng/ml TNF-α induced a significant increase in the release of IL-6 from human astrocytes (**Fig.** 5.7) (** $p<0.01$, versus control; one-way ANOVA, Bonferroni post-hoc test). This IL-6 release was significantly inhibited upon pre-treatment with FTY720-P (1μM) (** $p<0.01$, versus control; one-way ANOVA, Bonferroni post-hoc test) (**Fig.** 5.7). This result is interesting as it is evidence that S1P receptor activation may exert an anti-inflammatory effect on astrocytes with respect to IL-6 release. However the molecular mechanism by which S1P receptor activation exerts this effect remains unclear.

5.2.8 Activation of the S1P1 receptor by AUY954 causes a dose dependent inhibition of IL-6 release from human astrocytes.

Previous data has indicated that the inhibitory effect of FTY720-P on the release of pro-inflammatory signals from astrocytes is due to its interaction with the S1P1 receptor (Choi et al., 2011). To examine the role of the S1P1 receptors in FTY720-P mediated attenuation of IL-17/TNF-α induced IL-6 release, human astrocytes were pre-treated with increasing concentrations of the S1P1 receptor specific compound AUY954 prior to stimulation with a combination treatment of 50ng/ml IL-17 and 10ng/ml TNF-α. Serum starved, confluent human astrocytes were pre-incubated with 0.01-10nM AUY954. Following pre-incubation, a combination treatment of 50ng/ml IL-17 and 10ng/ml TNF-α was added to the astrocytes for an 18hr treatment at 37°C/5% CO2. Cell supernatant was then collected and IL-6 levels were measured by ELISA. Data showed that pre-treatment of human astrocytes with the S1P1 specific receptor agonist AUY954 prevented IL-17/TNF-α induced release of IL-6 (*** $p<0.01$ 0.1, 1 and 10nM AUY954 vs. IL-17/TNF-α) (**Fig.** 5.8). This provides evidence that the

previously observed effect of FTY720-P on cytokine induced IL-6 release is mediated primarily through the S1P1 receptor.

5.2.9 The IL-17 and TNF-α induced increase of IL-6 levels are not mediated via SphK activation.

FTY720 is phosphorylated by two conserved enzymes, sphingosine kinase 1 and 2 (SphK1 and SphK2) both of which play a major role in sphingolipid metabolism. Sphk2 has been found to be the enzyme predominately responsible for the phosphorylation of FTY720 *in vivo* (Billich et al., 2003). A number of studies have shown that SphK activity, in particular SphK1, is enhanced by the activation of many factors including TNF-α, PDGF (platelet derived growth factor), GDNF (glial derived neurotrophic factor), EGF (epidermal growth factor), FGF (fibroblast growth factor), VEGF (vascular endothelial growth factor), muscarinic acetylcholine agonists, and PKC (protein kinase C) (Dev et al., 2008; Maceyka et al., 2012). In addition the activation of Trk receptors (NGF activated Trk A, BDNF and NT 4/5 activated Trk B and NT3 activated Trk C), protease activated receptors (PARs) and Toll receptors (LPS activated TLR4) all induce SphK1 activity (Dev et al., 2008; Spiegel and Milstien, 2011). This activation of SphK1 increases levels of S1P, which is thought to activate the NF-κB signalling pathway intracellularly or transactivate S1P receptors extracellulary (Dev et al., 2008; Spiegel and Milstien, 2011). To investigate if IL-17 increased the release of a SphK pathway, the effects of a selective SphK1 inhibitor (SphK1i) was examined. A SphK1i, with an IC_{50} value of approximately 50nM and no activity on SphK2 at 10μM was synthesised as described previously (Xiang et al., 2010) (**Fig.** 9A). The data showed that the SphK1i had no effect on the control levels of IL-6 (**Fig.** 5.9B). Moreover, the SphKi did not alter IL-17 (**Fig.** 5.9B), or TNF-α induced (**Fig.** 5.9C) increase in IL-6 levels. These results suggest that increased intracellular S1P levels induced by the activation of SphK1, is not required for TNF-α or IL-17 mediated increase of IL-6 levels.

5.2.10 FTY720-P but not S1P causes S1P1 receptor internalisation in human astrocytes.

Studies show that FTY720-P binds to and causes rapid and sustained internalisation of membrane expressed S1P1 receptors (Mullershausen et al., 2009). Unlike S1P which causes recycling of the receptor, FTY720-P causes a persistent state of internalisation, followed by ubiquitination of the receptor and down-regulation at an mRNA level (Oo et al., 2007). This phenomenon has now been termed 'functional antagonism'. To examine the differential effects of S1P versus FTY720-P and AUY954 on IL-6 levels, the effects of these three compounds on S1P1 receptor internalisation in human astrocytes was determined. The data showed that, in contrast to S1P, treatment of astrocytes with FTY720-P and AUY954 induced robust and sustained internalisation of S1P1

receptors (**Fig.** 5.10). This data is in line with the hypothesis that both FTY720-P and AUY954 impede pro-migratory/pro-inflammatory response of the S1P-S1P1 receptor axis by internalisation and functional antagonism of S1P1 receptors.

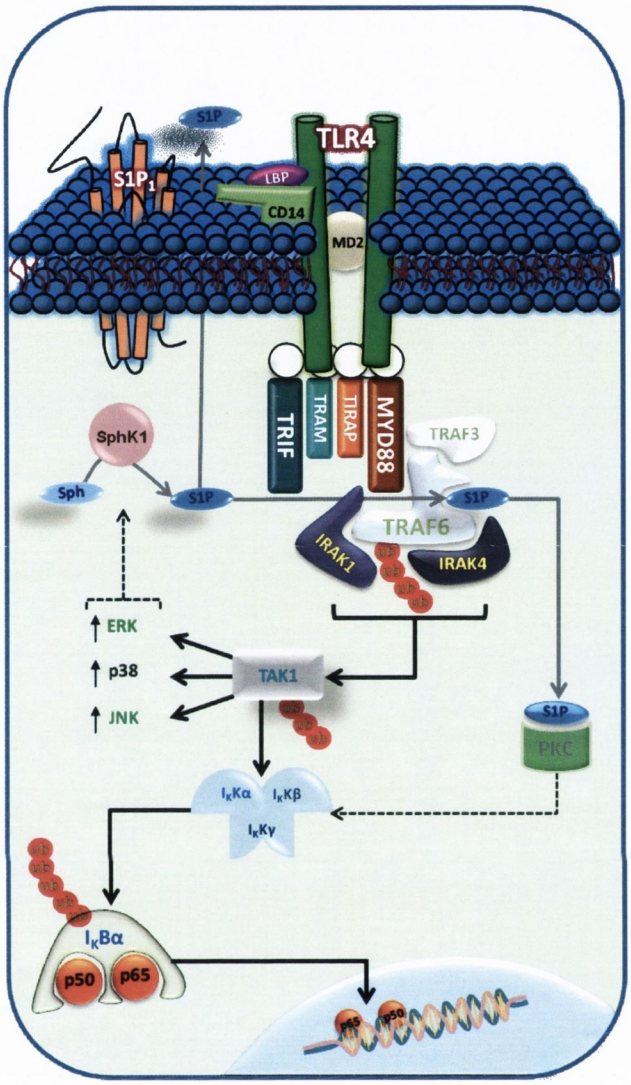


Figure 5.2 *The TLR4 signalling pathway with proposed sites of S1P involvement.*

This schematic outlines possible role of S1P in TLR4 mediated signalling. Dashed black line details possible S1P interactions. It has been shown that SphK1 mediated S1P generation leads to activation of PKC, which in turn promotes IKK and NF-κB activation (Puneet et al., 2010). It has also been suggested that S1P enhances the autoubiquitylation of TRAF6, which in turn activates TAK1 (Spiegel and Milstien, 2011). TAK1 phosphorylates the IKK complex, leading to the activation of NF-κB and MAPKs (Vallabhapurapu and Karin, 2009). S1P can be released to cross-activate S1P receptors in an autocrine fashion. In addition, it has been shown that TLR4 activation can lead to enchanced S1P1/3 receptor expression which may enchance pro-inflammatory cytokine expression, contributing to a positive feedback mechanism driving pro-inflammatory cytokine expression (Eskan et al., 2008).

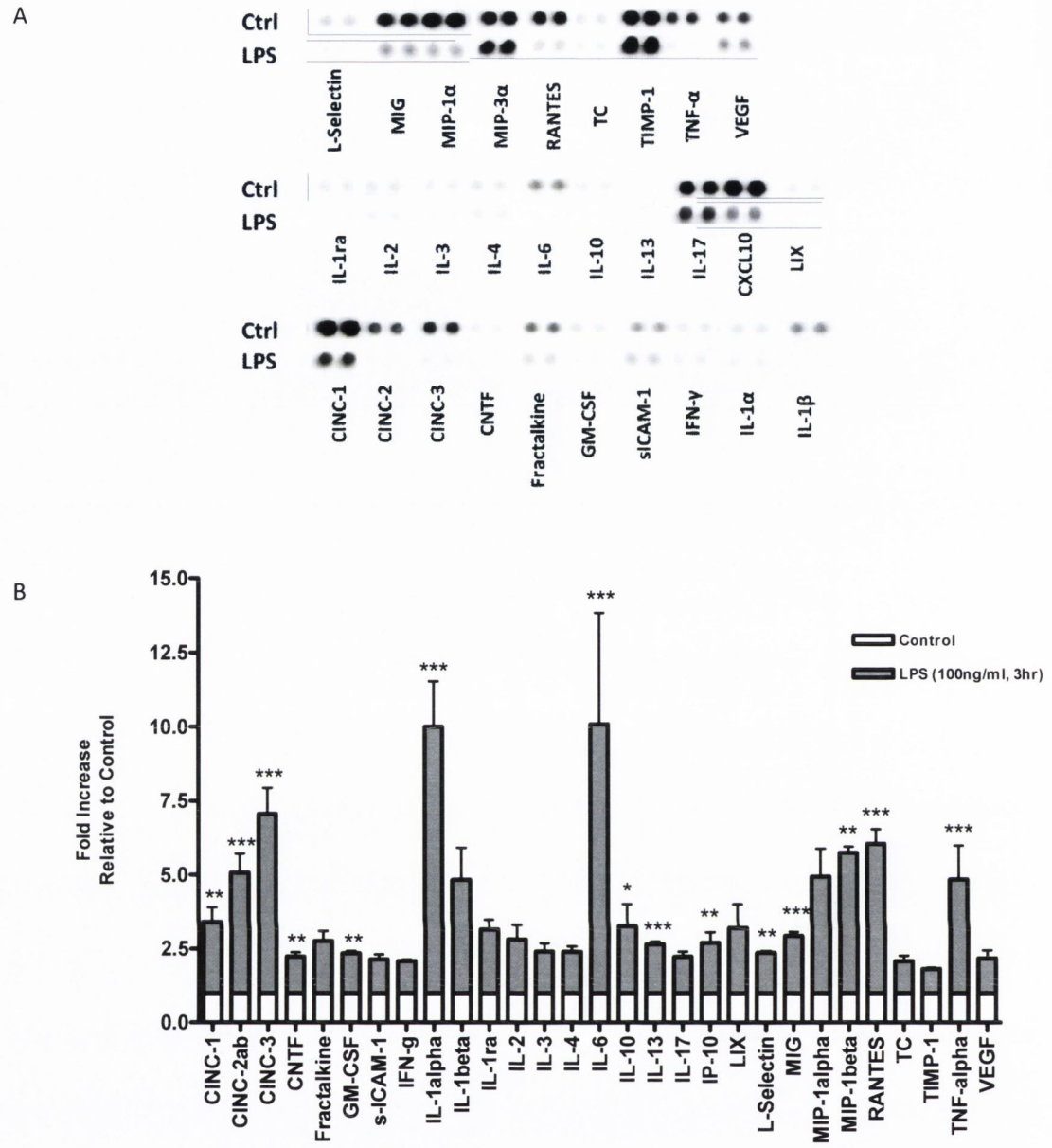


Figure 5.3 *LPS induces the release of a range of cytokines and chemokines from rat astrocytes.*

Primary rat astrocytes were treated with 100ng/ml LPS for 3hr. **(A)** Representative cytokine array blots. **(B)** Quantified data showed LPS induced a statistical significant increase in the release of chemokines and cytokines from astrocytes compared to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; as determined by unpaired t-test). Data represented as fold increase relative to control levels of cytokine/chemokine. (TC, Thymus Chemokine, CXCL10 (IP-10)). Values presented as means ± SD (n=2).

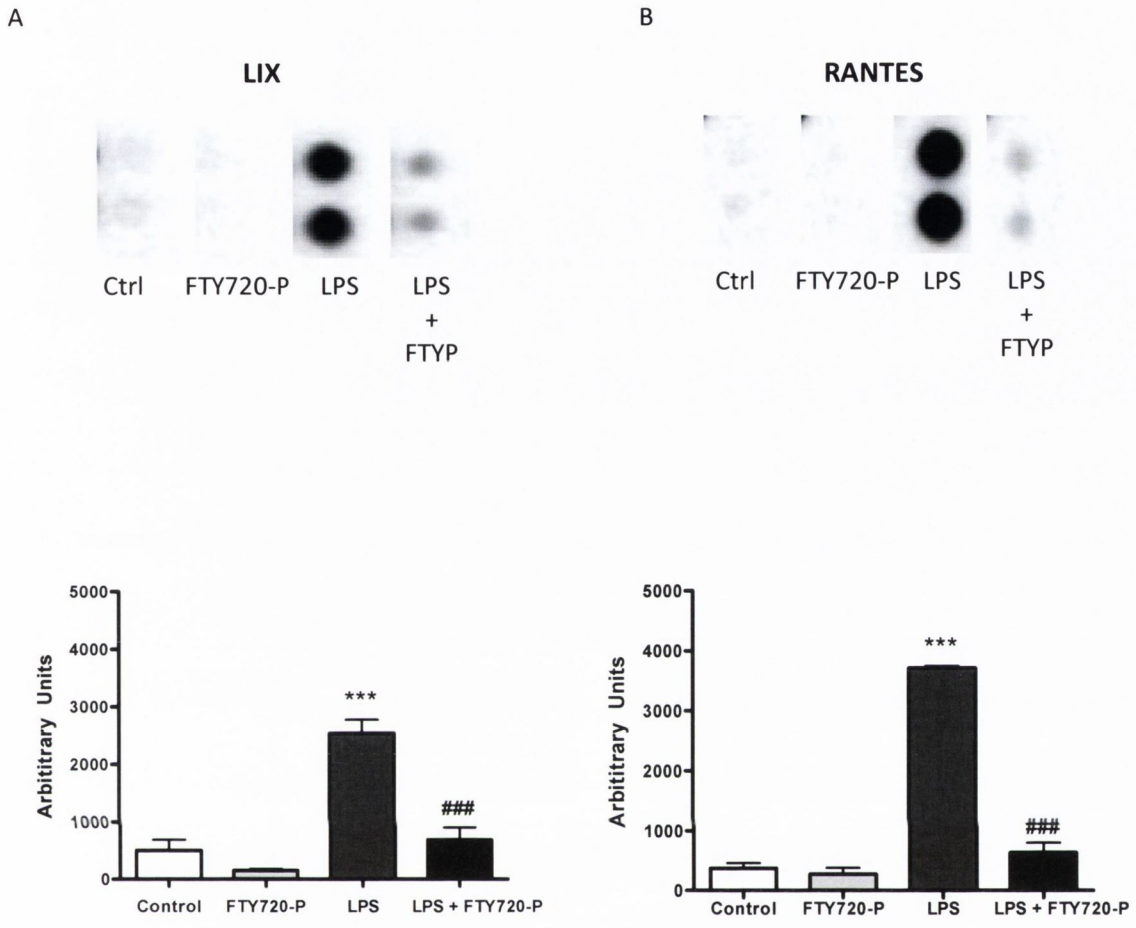


Figure 5.4 *FTY720-P treatment modulates LPS induced release of chemokines from rat astrocytes.*

Treatment with LPS (100ng/ml for 12hr) induced a statistically significant increase in release of both **(A)** LIX (CXCL5) and **(B)** RANTES (CCL5) from rat astrocytes (*** $p<0.001$ vs. control, one-way ANOVA, Bonferroni post-hoc test). Pre-treatment (1hr before LPS) with FTY720-P (1μM) significantly attenuated LPS induced LIX and RANTES release (### $p<0.001$ vs. LPS, one-way ANOVA, Bonferroni post-hoc test). Upper panels show representative cytokine array blots. Lower panels show quantified data. Y-axis values arbitrary. Values are represented as means ± SEM (n=3).

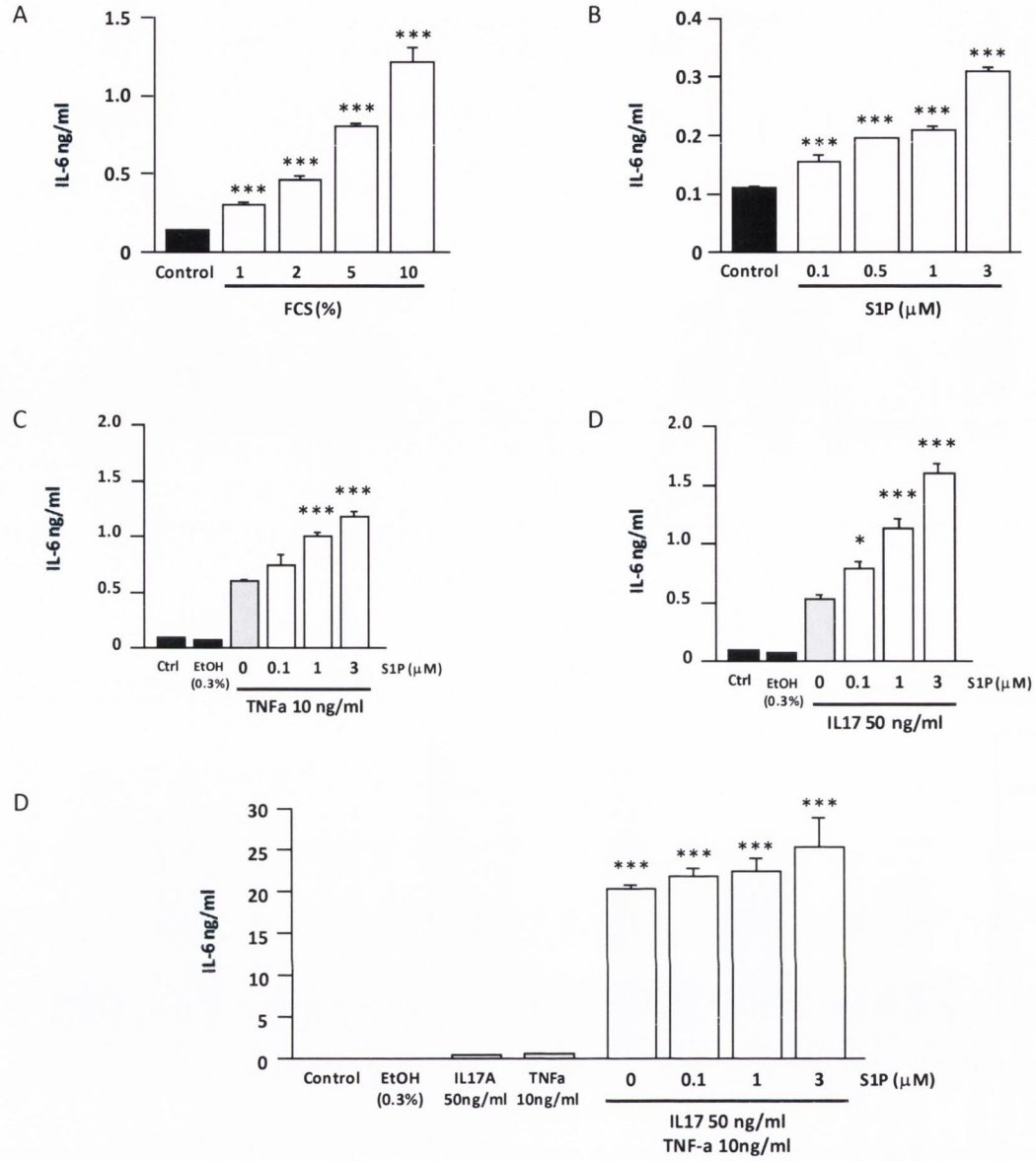


Figure 5.5 *S1P increases IL-6 levels in human astrocytes, in presence of IL-17 and/or TNF-α.*

Human astrocytes were plated (70,000 cells per well), grown for 3 days, serum starved for 4hr, after which they were treated for 20hr with increasing concentrations of **(A)** FCS and **(B)** S1P. In addition S1P treatments were done in combination with **(C)** 10ng/ml TNF-α, **(D)** 50ng/ml IL-17, and **(E)** IL-17 + TNF-α. All concentrations used are indicated in the figure. The protein levels of IL-6 in astrocyte conditioned media were examined by ELISA. Each condition was tested in triplicates. The data shown is a representative of 3 separate experiments. (*** $p<0.001$ vs. corresponding control; one way ANOVA, Dunnetts post-hoc test). Data generated by Elain Gaelle (NIBR, Basel, Switzerland).

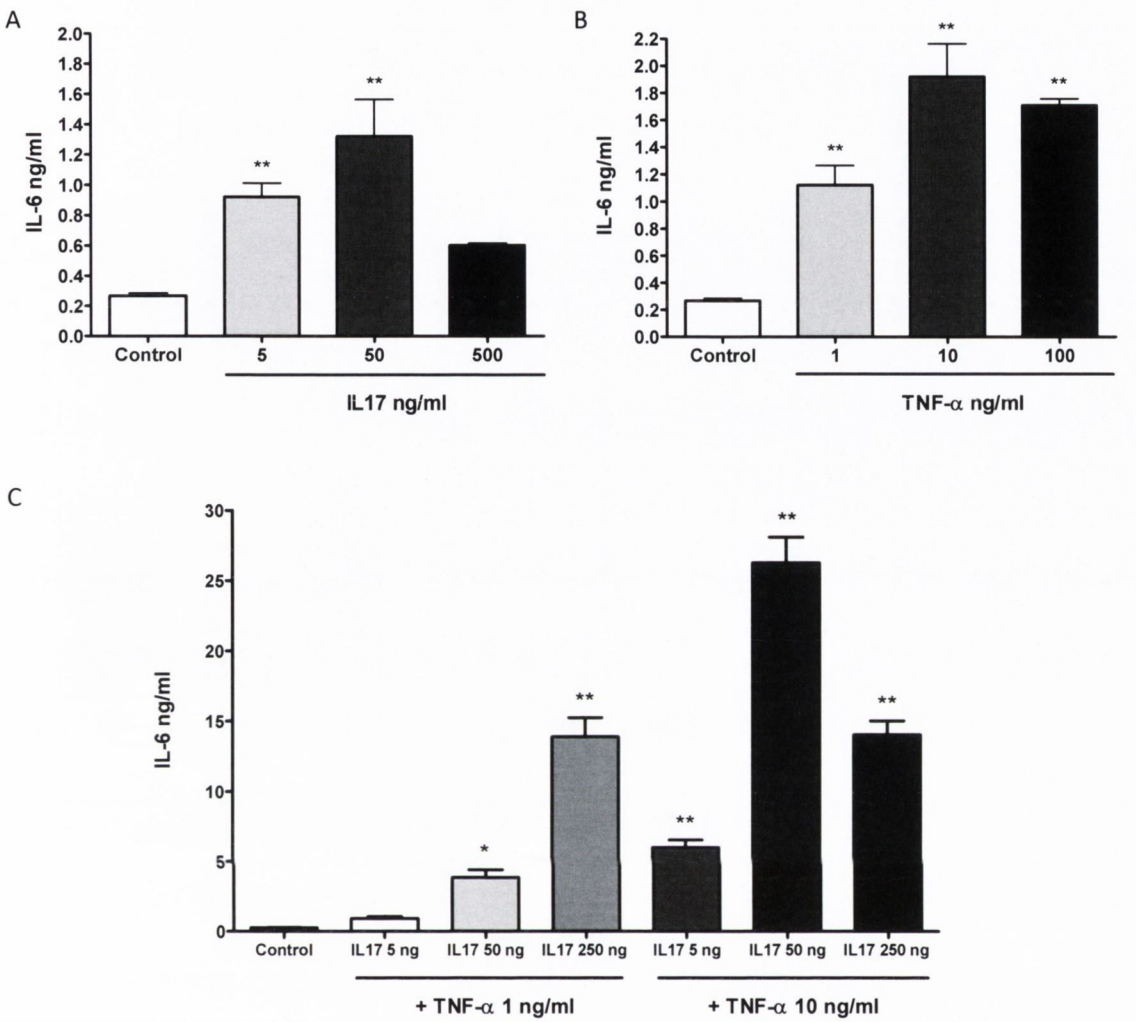


Figure 5.6 *Synergistic effect of IL-17 and TNF-α signalling enhances the release of IL-6 from human astrocytes.*

Serum starved human astrocytes were treated with **(A)** human recombinant IL-17 (5-500ng/ml) for 18hr. Astrocytes were also treated with **(B)** human recombinant TNF-α (1-100ng/ml). Both treatments resulted in the release of significant levels of IL-6 as measured by ELISA (** $p<0.01$, one-way ANOVA, Dunnett's post-hoc test). **(C)** Serum starved astrocytes were treated with various combinations of cytokines to determine the optimal combination for induction of IL-6 release. An IL-17/TNF-α combination of 50/10ng/ml induced the most significant release of IL-6 from human astrocytes as measured by ELISA. Statistical analysis was conducted using one-way ANOVA and Bonferroni post-hoc test. Values are represented as means ± SEM (n=2).

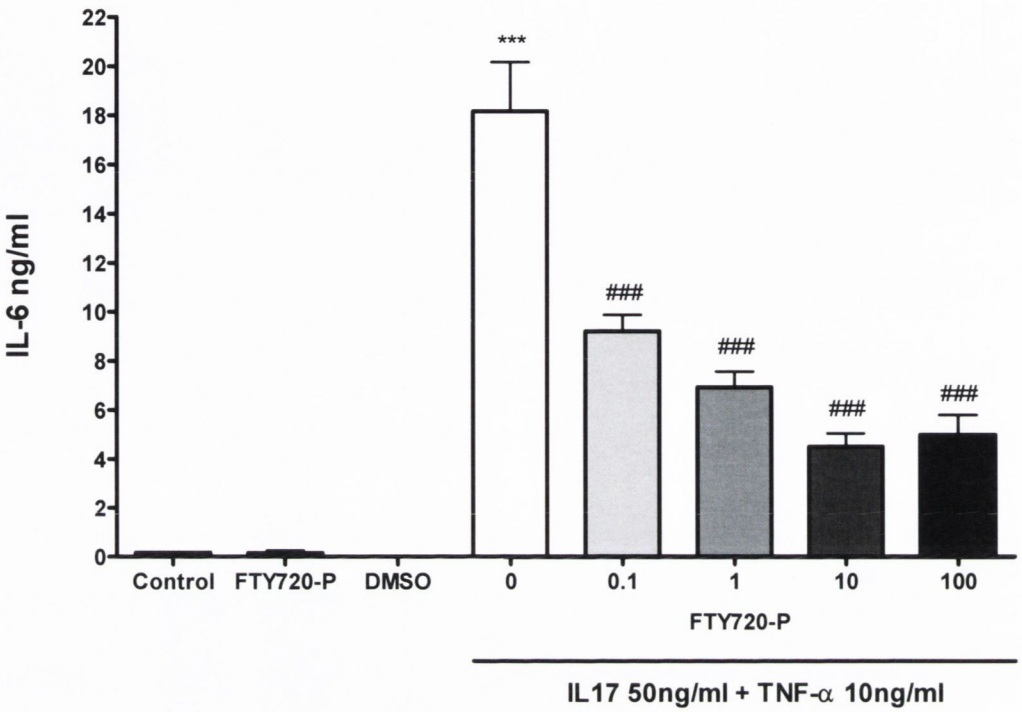


Figure 5.7 *Pre-treatment of human astrocytes with FTY720-P causes a dose dependent inhibition of IL-6 release.*

Serum starved human astrocytes were pre-treated with FTY720-P for 1hr prior to stimulation of the cells with a combination of IL-17 and TNF-α (50/10ng/ml) for 18hr. Supernatant was examined by way of ELISA for the release of IL-6. Cytokine treatment induced a significant release of IL-6 (*** $p<0.001$, IL-17/TNF-α vs. control; one-way ANOVA, Bonferroni post-hoc test). FTY720-P pre-treatment of human astrocytes ameliorated the cytokine induced release of IL-6 (### $p<0.001$, FTY720-P treatment vs. IL-17/TNF-α; one-way ANOVA, Bonferroni post-hoc test). Values are represented as means ± SEM (n=3).

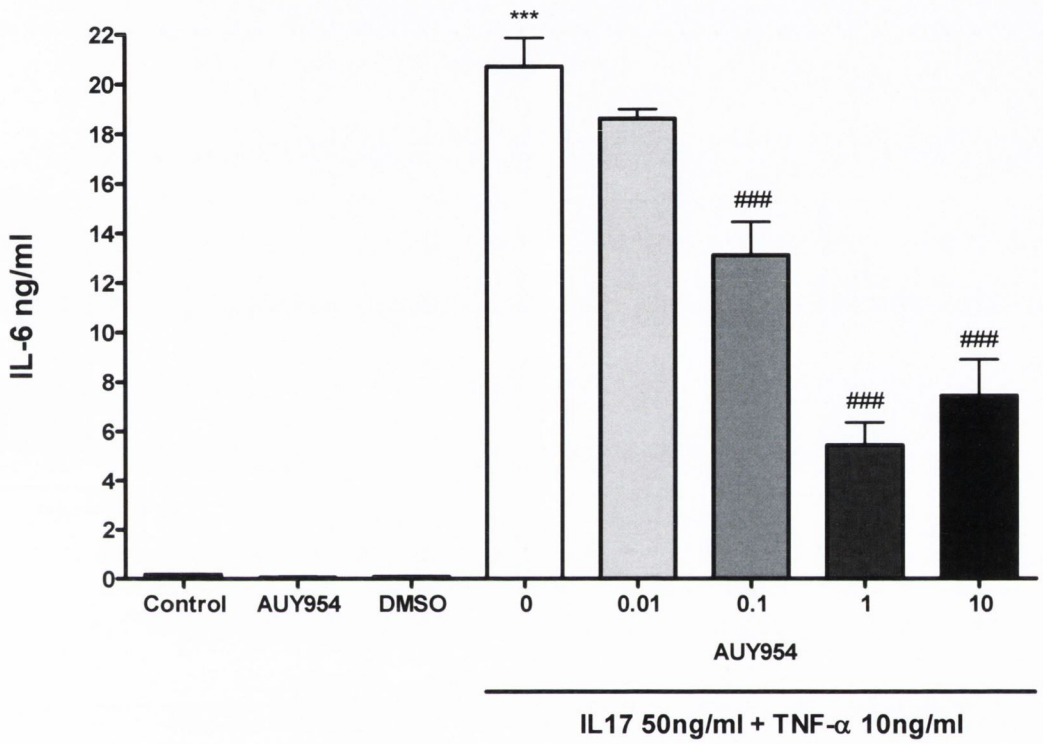


Figure 5.8 *Activation of the S1P1 receptor by AUY954 causes a dose dependent inhibition of IL-6 release from human astrocytes.*

Serum starved human astrocytes were pre-treated with AUY954 for 1hr prior to stimulation of the cells with a combination of IL-17 and TNF-α (50/10ng/ml) for 18hr. IL-17/TNF-α treatment induced a significant release of IL-6 from astrocytes (*** $p<0.001$, IL-17/TNF-α vs. control; one-way ANOVA, Bonferroni post-hoc test). Supernatant was examined by way of ELISA for the release of IL-6. AUY954 pre-treatment of human astrocytes ameliorated the cytokine induced release of IL-6 (### $p<0.001$, AUY954 treatment vs. IL-17/TNF-α one-way ANOVA, Bonferroni post-hoc test). Values are represented as means ± SEM (n=3).

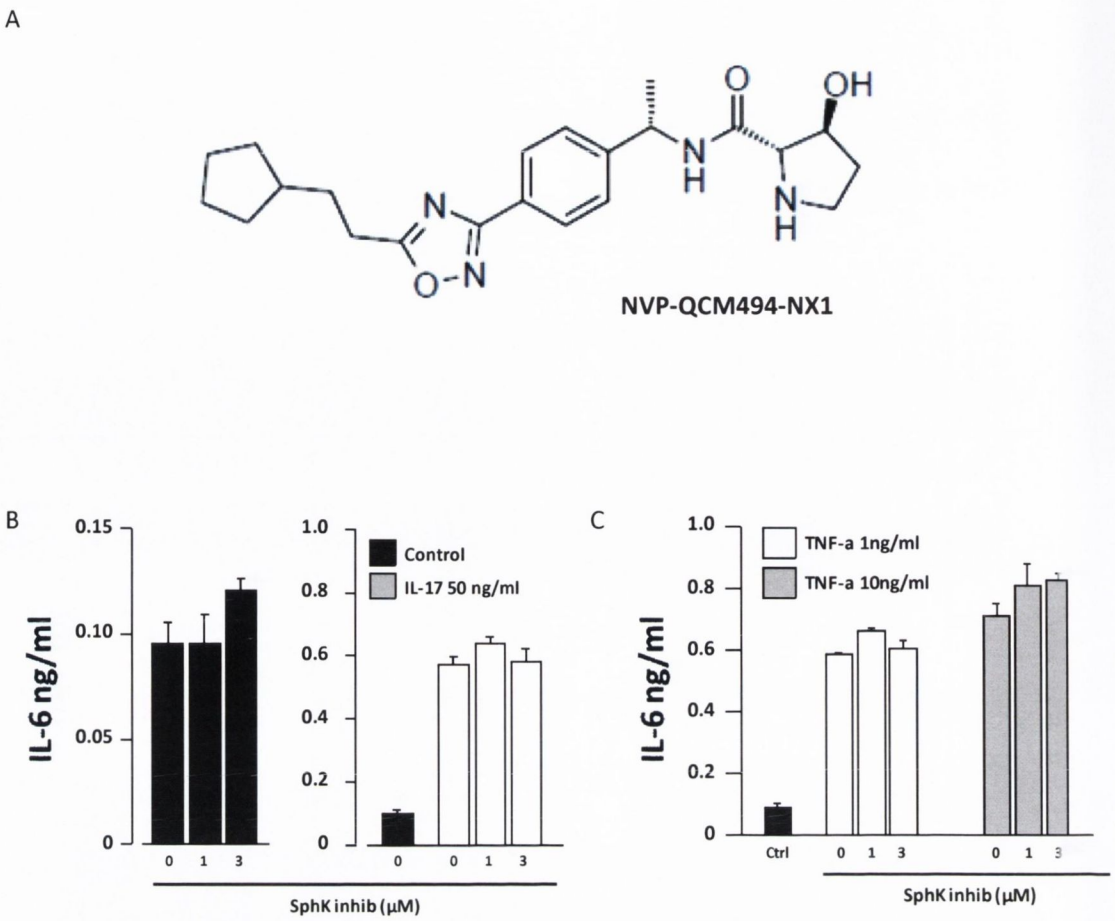


Figure 5.9 *The IL-17 and TNF-α induced increase of IL-6 levels is not mediated via SphK activation.*
The structure of the SphK inhibitor (SphKi) synthesised is shown **(A)** and is reported to inhibit SphK1 with an IC_{50} of 58nM, while being inactive on SphK2 at 10μM. Primary human astrocytes were plated at 70,000 cells in 24 well plates, grown for 3 days, serum starved for 4 h, after which they were pre-treated for 2hr with increasing concentrations of SphKi followed by treatment with **(B)** IL-17 (50ng/ml), or **(C)** TNF-α (1 or 10ng/ml) for 18hr. All concentrations used are indicated in the figure. The protein levels of IL-6 in astrocyte conditioned media were examined by ELISA. Each condition was tested in triplicates. The data shown is a representative of 3 separate experiments. Data generated by Elain Gaelle (NIBR, Basel, Switzerland).

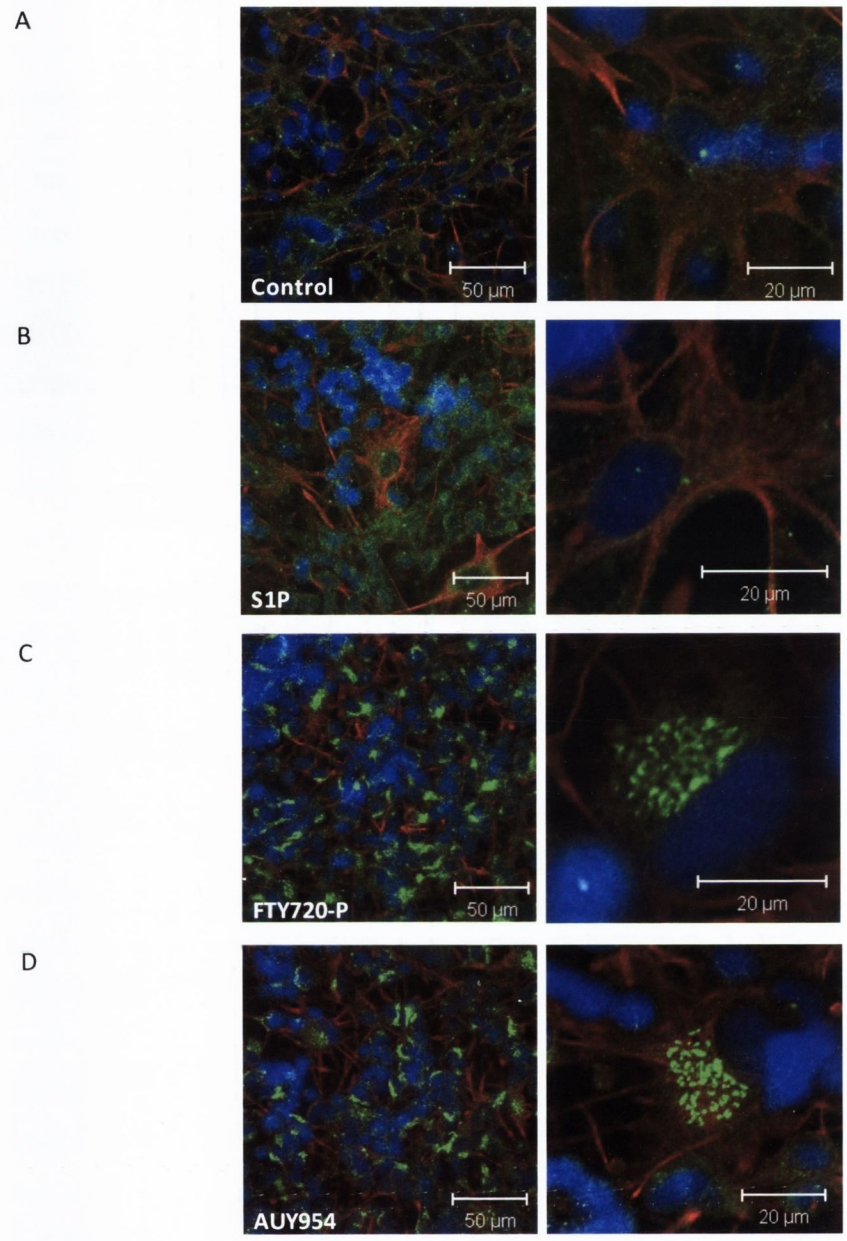


Figure 5.10 *FTY720-P but not S1P causes S1P1 receptor internalisation in human astrocytes.*

Treatment of cultured human astrocytes with **(A)** DMSO (0.1%) or **(B)** S1P treatment (1µM for 1hr) had no effect on S1P1 receptor internalisation states. In contrast, treatment with **(C)** FTY720-P (1µM for 1hr) **(D)** AUY954 (1µM for 1hr) caused internalisation of S1P1 receptors to a perinuclear region. In S1P1 receptor (green), GFAP (red), Hoechst (blue), images on the right are example single cells taken 63x magnification. Data shown is representative of 2 independent experiments.

5.4 Discussion

To further examine the function of S1P1 receptors in the CNS, their role in cytokine release from human astrocytes was examined. Here, focus was given to the release of two chemokines LIX and RANTES, released from rat astrocytes, and the release of IL-6 from human astrocytes. Both LIX and RANTES release and expression of their respective receptors are involved in the trafficking and distribution of circulating leukocytes which is clinically relevant for diseases such as MS (Ransohoff, 1999). IL-6 release is implicated in a number of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and MS (Neurath and Finotto, 2011). In the CNS, IL-6 contributes to a pro-inflammatory milieu, mainly through the support of pro-inflammatory Th17 cells and suppression of anti-inflammatory T-regulatory cells (Kimura and Kishimoto, 2010). The data showed that *in vitro* treatment of rat astrocytes with the bacterial endotoxin LPS increased the levels of a wide range of cytokines and chemokines. Release of LIX and RANTES was attenuated by pre-treatment with FTY720-P. Following on from this, the data showed that both IL-17 and TNF-α can independently induce the release of pro-inflammatory cytokine IL-6 from human astrocytes. A combination of both IL-17 and TNF-α induced the release of a significant level of IL-6, greater than an additive effect of the two cytokines. This synergistic signalling of IL-17 and TNF-α was inhibited by pre-treatment with FTY720-P. S1P1 receptor involvement in this attenuation of IL-6 release was demonstrated by data showing the S1P1 receptor specific agonist AUY954 inhibiting cytokine induced IL-6 release. Importantly, the *in vitro* stimulation of human astrocytes with S1P significantly increased the levels of IL-6. This was in contrast to the effect of FTY720-P and AUY954. These effects were not associated with involvement of the sphingosine kinase pathway. Both FTY720-P and AUY954, but not S1P, internalised S1P1 receptors suggesting that the effects on FTY720-P and AUY954 on reducing IL-17/TNF-α mediated increase of IL-6 were likely due to S1P1 receptor functional antagonism. Taken together, the data suggested that FTY720-P attenuates the release of pro-inflammatory cytokines and chemokines from both rat and human astrocytes via a mechanism that involves internalisation and functional antagonism of S1P1 receptors.

5.4.1 The role of IL-17/ TNF-α signalling in astrocytes.

Astrocytes have diverse roles that S1P1 receptors may regulate, including (i) direct communication of astrocytic end-feet with endothelial cells that allows astrocytes to control the blood-brain-barrier; (ii) uptake of neurotransmitters, such as glutamate, at the synaptic cleft that allow astrocytes to regulate synaptic transmission and excitoxicity; and (iii) release of growth factors and cytokines, that allow astrocytes to regulate cellular communication, migration and survival, for example of neurons

and lymphocytes. It is significant that astrocytes form scar pathology after CNS injury, thus having apparent opposing roles in physiology and pathophysiology. In disease, astrocytes are suggested to play roles in a range of psychiatric, neurological and neurodegenerative disorders (Sidoryk-Wegrzynowicz et al., 2011). Novel drugs that alter astrocyte function are thus of great potential value, where regulation of astrocyte function by S1P1 receptors may prove therapeutically useful for a range of CNS diseases. We have shown previously that astrocytes express S1P receptors and their activation regulates many intracellular signalling cascades, including increased ERK phosphorylation (pERK), elevated Ca^{2+} levels, inhibition of adenylyl cyclase and activation of PLA_2 and PLC (Mullershausen et al., 2007; Osinde et al., 2007). We also find that S1P1 receptor activation promotes astrocytes migration, in contrast to FTY720-P limiting T-cell migration, but in line with S1P induced migration of neural stem cells and oligodendrocyte precursors (Kimura et al., 2007; Miron et al., 2008a; Mullershausen et al., 2007). Importantly, S1P promotes the release of growth factors (NGF, FGF-2, and GDNF) from astrocytes that can allow for cell-crosstalk and promote neuronal survival (Furukawa et al., 2007; Sato et al., 1999; Yamagata et al., 2003). Astrocytes secrete a vast arrays of signalling molecules including neurotransmitters, neuropeptides, growth factors and members of all the three families of cytokines, namely lymphokines, interleukins and chemokines (both CCL and CXCL), many of which are located in active areas of MS lesions (Dong and Benveniste, 2001; Qin and Benveniste, 2012; Tzartos et al., 2008). Previous studies have shown that astrocytes express both IL-17RA and IL-17RC and they respond to IL-17, via IL-17/Act1 signalling, to produce a number of pro-inflammatory cytokines including IL-6, TNF-α and IL1β, that induce granulocyte and leukocyte recruitment (Das Sarma et al., 2009; Graber et al., 2008; Kang et al., 2010; Ke et al., 2009; Nichols et al., 2009). Of note, synergistic effects of IL-17 and TNF-α or IL1β have shown to be mediated via the NF-κB pathway as determined by use of inhibitors of NF-κB (BAY-11) (Ma et al., 2012; Meares et al., 2012; Trajkovic et al., 2001). Additionally, IL1β, IL-6 and IL-17 are increased during EAE and specific S1P1 receptor knockout from astrocytes or FTY720-P treatment reduces their release (Choi et al., 2011). In agreement with these previous studies, the data here showed that IL-17 induced an increase in level of IL-6 in human astrocytes and that the levels of IL-6 were greatly increased in astrocytes co-treated with IL-17 and TNF-α. The release of cytokines from astrocytes likely results in a wave of leukocyte infiltration into the CNS, which in turn propagates and exacerbates the inflammatory event (**Fig.** 5.11). Thus it is highly significant that FTY720-P and the selective S1P1 receptor ligand, AUY954, prevented IL-17 induced increase in levels of IL-6 in human astrocytes. These studies support the hypothesis that shutdown of communication between

astrocytes and immune cells is part of the mechanism by which FTY720-P shows efficacy in reduction of relapse rates in MS.

5.4.2 Functional Antagonism versus Receptor Agonism.

The experiments described in this chapter support the notion that agonism of CNS derived S1P1 receptors as opposed to functional antagonism (as seen in T-cells) is responsible for the neuroprotective effects of FTY720-P. The "functional antagonism theory" suggests that cross activation of plasma membrane bound S1P1 receptor by receptors for pro-inflammatory molecules leads to the expression of pro-inflammatory cytokines and chemokines. Therefore pre-treatment with FTY720-P results in functional antagonism of the S1P1 receptors, resulting in a lack of plasma membrane bound receptor and thus preventing TLR4/IL-17/TNF-α receptor mediated cross-activation (**Fig.** 5.12, Hypothesis I). This theory however would involve SphK1 induced production of S1P, subsequent release of S1P and S1P activation of plasma membrane expressed S1P receptors. Studies have shown increased expression of SphK1 in astrocytes of MS lesions (Fischer et al., 2011) and indicate that the SphK1/S1P1 axis, in particular, can inhibit LPS induced pro-inflammatory cytokine/chemokine production (Schroder et al., 2011). The current study however indicates that IL-17/TNF-α induced release of IL-6 does not involve the activation of SphK1 (**Fig.** 5.9). It is also possible that activation of IL-17 receptors may promote the surface expression of TNF-α receptors, thus enhancing TNF-α induced IL-6 release; this in turn may be inhibited by S1P receptor downstream signalling events (**Fig.** 5.12, Hypothesis II). However it is more likely that both IL-17 and TNF-α signal to a common downstream second messenger which could be inhibited by direct S1P receptor agonism. The common second messenger system is likely to be the NF-κB pathway. Specifically, direct agonism of the S1P1 receptor by FTY720-P likely causes a downstream inhibition of TLR4 or IL-17/TNF-α mediated NF-κB signalling (**Fig.** 5.12, Hypothesis III). Indeed studies have shown that selective knockdown of the NF-κB signalling pathway in astrocytes leads to reduced white matter damage and increased functional recovery following contusive spinal cord injury (Brambilla et al., 2005). These effects correlated with reduced expression of pro-inflammatory cytokines and chemokines, and in the particular CCL2 and CXCL10 (IP-10) (Brambilla et al., 2005). In addition it has been shown that CNS-restricted ablation of the NFκB pathway is protective in an EAE setting (van Loo et al., 2006). We hypothesise that S1P/S1P receptor attenuation of cytokine induced pro-inflammatory cytokine and chemokine release involves signalling to the NF-κB pathway (**Fig.** 5.2).

5.4.3 Conclusion and Future Work.

Due to inappropriate immune responses during inflammatory/autoimmune diseases such as MS, recruitment of leukocytes into the brain parenchyma and inflammatory lesions by chemokines (such as those derived from astrocytes) may serve to potentiate chronic inflammation. This in turn can play a crucial role in the onset and maintenance of the observed pathophysiology (Ransohoff, 1999). Beyond infiltration, chemokines, such as those described here, serve to directly alter function of astrocytes and other cells within the CNS (Nair et al., 2008). Future work aimed at characterising and modulating the cytokine and chemokine networks may provide valuable therapeutic options. Indeed, such approaches have already been shown to be efficacious in the treatment of MS, where IVIGs were shown to differentially alter expression of a number of chemokines in patients with active MS (Pigard et al., 2009). Astrocytes are triggered to release cytokines and chemotactic factors, following stimulation with bacterial and pathogen-related products, components of the clotting cascade, lymphocyte-derived factors, and interactions with a wide range of immune cell infiltrates (Sofroniew and Vinters, 2010). Subsequent to the observations of FTY720-P mediated attenuation of LPS and IL-17/TNF-α induced reactivity in primary astrocyte cultures, the observed efficacy of FTY720-P in the treatment of MS may be considered to incorporate alternative neuroprotective mechanisms. Apart from its immunological properties FTY720-P may act through the induction of anti-inflammatory signalling or the desensitization of astrocytes to inflammatory stimuli thereby having an inhibitory effect on astrogliosis. The exact profile of chemotactic molecules released is dependent on various parameters including expression of the TLR4, IL-17 or TNF receptors, activation state of the astrocytes etc. These findings further support the notion that the efficacy of FTY720-P is dependent on effects in both the immune system and CNS, through its inhibition of chemotactic signals and subsequent 'dampening' of an autoimmune response. The data also supports the testing of FTY720-P in animal models of other neuroinflammatory, neurodegenerative and demyelinating conditions, beyond MS.

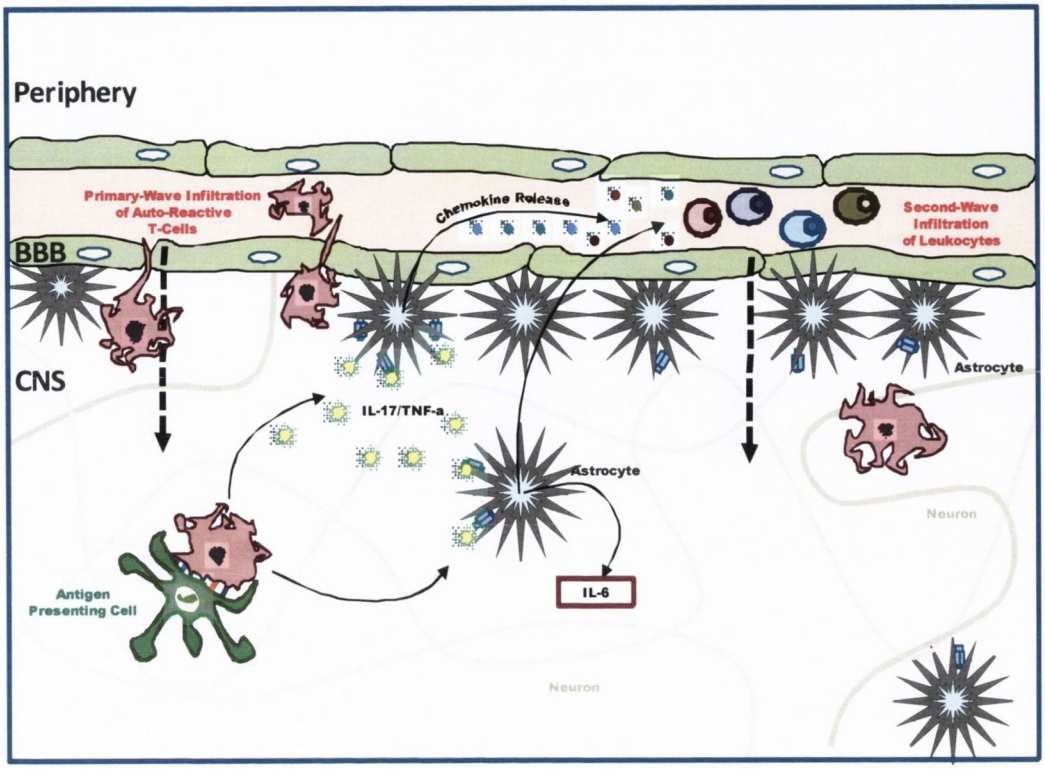


Figure 5.11 *Th1-Th17 mediated neuroinflammation via astrocytic activation.*

Drainage of soluble self-antigens from the CNS parenchyma into cervical lymph nodes has been demonstrated (de Vos et al., 2002). This may cause low intensity cross-activation of T_{CM} cells, these cells then re-circulate and enter the CNS, possibly at the choroid plexus where they cross the blood-CSF barrier (Reboldi et al., 2009). Once in the CNS parenchyma they are re-stimulated by local dendritic/microglial cells causing them to proliferate and differentiate into T_{EM} cells and subsequently release pro-inflammatory cytokines (Sospedra and Martin, 2005). Cytokines act on neighbouring astrocytes through their cognate receptors to stimulate the release of chemotactic molecules which attract a second wave of leukocytes which actively drives a highly damaging inflammatory cascade (Kang et al., 2010).

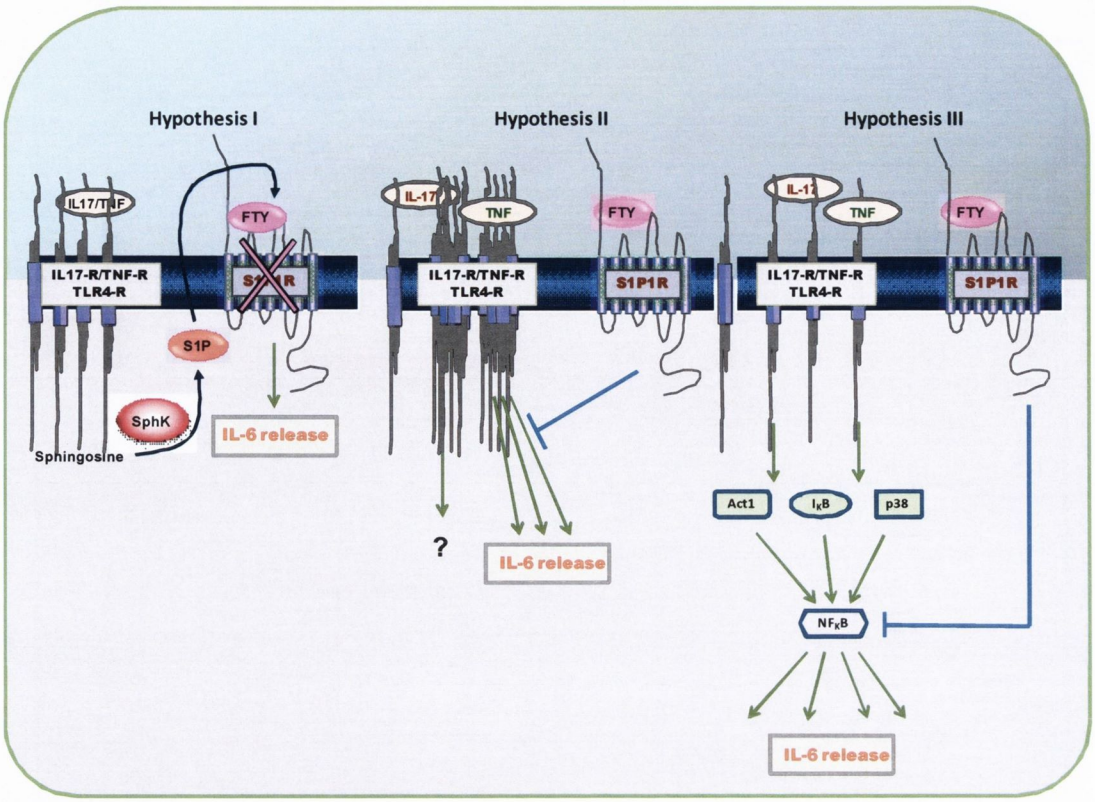


Figure 5.12 *FTY720-P mediated inhibition of pro-inflammatory cytokine release: Hypotheses.*
We show that pre-treatment with FTY720-P significantly reduces the release of the pro-inflammatory cytokine IL-6 from human astrocytes stimulated with IL-17 and TNF-α (**Fig.** 5.7). Here we hypothesise as to the mechanism by which FTY720-P exerts this effect. 'Hypothesis I' involves the release of S1P in response to cytokine activation which then acts on membrane bound S1P receptors which drives IL-6 release, this is blocked by the functional antagonistic effect of FTY720-P on these receptors. 'Hypothesis II' suggests that activation of IL-17 receptors may promote the surface expression of TNF-α receptors thus enhancing TNF-α induced IL-6 release. This in turn may be inhibited by S1P receptor downstream signalling events. 'Hypothesis III' indicates a role for a common downstream second messenger between the IL-17 and TNF-α signalling pathways which could be inhibited by direct S1P receptor agonism. This figure represents hypothetically the mechanism by which FTY720-P exerts its anti-inflammatory effect on cytokine stimulated human astrocytes.

Chapter 6. Discussion

6.1 Discussion

6.1.1 Opening Remarks.

S1P1 receptors expressed on T-cells are widely recognized as the primary molecular targets responsible for Gilenya's efficacy in the treatment of RRMS. It is thought that FTY720-P mediated functional antagonism of these GPCRs on T_{CM}'s and the resultant peripheral lymphopenia is the key molecular event responsible for the drug's therapeutic effect (Mehling et al., 2008). While this probably remains the case it would be remiss to rule out a direct CNS effect. It remains highly unlikely given the lipophilic nature of the compound, coupled with the widespread expression of its cognate receptors, that FTY720-P's interaction with CNS located S1P receptors does not contribute to its clinical efficacy in the treatment of RRMS. Numerous recent publications have shed a light on astrocytic involvement in the complex process of neuroinflammation (Choi et al., 2011; Kang et al., 2010). Reactive gliosis was long considered to be astrocyte's only real involvement in neuroinflammation. However recent studies have provided evidence that astrocytes play a central role in the progression of EAE (Kang et al., 2010). These studies show that through interactions with Th17 lymphocytes, astrocytes provide chemotactic signals for leukocytes, promoting their entry to the CNS compartment (Kang et al., 2010). Data from astrocyte specific S1P1 receptor knockout animals shows that FTY720-P mediates a non-immunological, CNS mechanism of action (Choi et al., 2011). This study also shows that both genetic knockout of the S1P1 receptor from astrocytes, and pharmacological loss of CNS S1P1 receptors results in a reduction in EAE clinical scores (Choi et al., 2011). This suggests that it is FTY720-P induced persistent internalisation of S1P1 receptors on astrocytes in agreement with what we observe (**Fig.** 3.5) and not a persistent agonism (**Fig.** 4.11) that mediates the compounds protective effect in an EAE setting. It is feasible that functional antagonism of the S1P1 receptor on astrocytes could be beneficial in reducing the overall activation of astrocytes and reducing the possibility of gliosis. Thereby reducing production of inflammatory mediators by preventing either S1P mediated activation (as local S1P production is markedly increased in EAE and MS) of membrane bound S1P1 receptor or preventing its cross activation by other receptors. However our data shows FTY720-P mediated inhibition of LPS, IL-17 and TNF-α induced release of chemokines and cytokines, suggesting a more direct agonistic effect of FTY720-P. It is possible that a combination of FTY720-P induced agonism resulting in downstream signalling events coupled with an overall loss of receptor from the cell surface leads to an inhibition in pro-inflammatory cytokine production while preventing induction of an activated astrocytic phenotype.

6.1.2 Internalisation of Astrocytic S1P1 Receptors- Results Chapter 1.

In the literature a conclusion has yet to be drawn on the exact molecular events governing S1P1 receptor internalisation. Some groups provide evidence for a clathrin-independent system with caveolae involvement (Igarashi and Michel, 2000). However the majority of studies suggest the more classical route of GPCR internalisation via clathrin-coated pits (Watterson et al., 2002). The cyclic process that accompanies ligand-GPCR interaction is designed to prevent cells from undergoing excessive receptor signalling and generally follows the route of ligand binding, receptor signalling, desensitisation, internalisation, resensitisation and recycling back to the plasma membrane (Hanyaloglu and von Zastrow, 2008). Such is the case when S1P binds to the S1P1 receptor (**Fig.** 3.7). The receptor signals, is internalised to the endosomal compartment where a change in vesicular pH causes S1P to be stripped from the GPCR (Rosen et al., 2009). Under normal conditions most of the receptor molecules are trafficked to sorting endosomes and then recycled back to the plasma membrane with a half-life of approximately 30mins (Rosen et al., 2009). Binding of FTY720-P to the S1P1 receptor results in its persistent internalisation to the Golgi complex and eventual down-regulation (**Fig.** 1.7) (Mullershausen et al., 2009).

Following activation, GPCRs are subject to various modifications including phosphorylation, palmitoylation and ubiquitination (Brady and Limbird, 2002; Marchese et al., 2003). Although both S1P and FTY720-P are full agonists of the S1P1 receptor, their binding to the GPCR results in separate cell fates, which are likely due to different receptor ubiquitination states. There is a strong correlation between levels of ubiquitinylation and targeting of receptors for proteasomal degradation (Shenoy et al., 2001). Monoubiquitylation mediates GPCR interactions with the protein machinery that is involved in endosomal trafficking, in contrast to polyubiquitylation, which serves to target proteins to the proteasome for degradation (Shenoy et al., 2001). FTY720-P stimulated S1P1 receptor results in receptor polyubiquitylation, whereas S1P bound S1P1 receptor results in monoubiquitylation (Oo et al., 2007). It is not clear thus far what causes this difference in ubiquitination states. It has been shown that FTY720-P bound S1P1 receptors differ in structure to S1P bound S1P1 receptors (Hanson et al., 2012; Parrill et al., 2012). It is possible that the structural difference in the ligand bound S1P1 receptor alters its level of post-translational modification. Certain E3 ubiquitin ligases may recognise the receptor conformation induced by FTY720-P bound S1P1 receptor as opposed to that induced by S1P; this may lead to aberrant receptor ubiquitinylation states. However it is also worth noting that these ubiquitinylation differences may also be due to a difference in ligand-receptor stability. S1P is rapidly degraded within the cell and easily stripped from the S1P1 receptor thus leaving the receptor free to be trafficked back to the cell surface for further activation (Hla and Brinkmann, 2012). FTY720-P however binds tightly to the S1P1

receptor and the resulting polyubiquitylation may target the receptor to the Golgi complex, from there the receptor may be degraded and down regulated. Several studies have shown that deletion of the extreme c-terminus of the S1P1 receptor caused inhibition of induced internalisation (Oo et al., 2011; Watterson et al., 2002). These studies led us to design a peptide (MNP301) to block putative protein-protein interactions at the c-terminus of the receptor with the objective of interfering with its internalisation. We show through the use of MNP301 that putative interactions at the c-terminus of the S1P1 receptor may be crucial for the FTY720-P induced persistent internalisation of the receptor to the TGN/Golgi matrix (**Fig.** 3.10). The identification of some of these interacting proteins will be an important feature of future work on this project.

6.1.3 Pathway Specific Antagonism by MNP301-Results Chapter 2.

We show that modulation of the c-terminus of the S1P1 receptor alters the receptor's persistent signalling properities in a pathway specific manner; the consequences of these findings warrants further investigation. Analysis of S1P1 G_i-mediated receptor signalling following internalisation confirms that the GPCR continues to signal to the AC/cAMP pathway once internalised (Mullershausen et al., 2009). We find that the blocking peptide MNP301 does not interfere with the S1P1 receptors ability to inhibit fsk-induced production of cAMP (**Fig.** 4.11). Post-endocytotic GPCR signalling is not a new concept with strong evidence existing for the activation of the MAPK cascade by the β_2-adrenergic receptor from the endosomal compartment (McDonald et al., 2000). This phenonenon is relevant in terms of explaining the functional antagonism versus persistent signalling of s1P1 receptors in relation tp the efficacy of Gilenya®. It is tempting to speculate that MNP301 alters the cellular fate of internalised FTY720-P bound S1P1 receptors. In parallel to cAMP levels we also examined S1P receptors ability to induce Ca^{2+} signals. We show that MNP301 specifically inhibits S1P1 receptor induced Ca^{2+} signalling but not cAMP levels (**Fig.** 4.6). These experiments provide evidence of a pathway specific effect of MNP301 on S1P1 receptor signalling. Similar to its effect on S1P1 receptor internalisation we suggest that MNP301 prevents the S1P1 receptor from interacting with a secondary complex that is involved in coupling S1P1 receptors to the PLC/IP3 pathway, thereby inhibiting Ca^{2+} signalling. This may or may not be the same complex associated with FTY720-P induced receptor internalisation. In contrast the c-terminal may not be crucial in coupling the S1P1 receptor to the AC/cAMP pathway. Identification of the proteins involved in this putative complex represents a key aim for future work in this field.

6.1.4 The Release of Pro-inflammatory Cytokines and Chemokines-Results Chapter 3.

Chemokines are small (7-10 kDa) chemotaxis inducing molecules (Kehrl, 2006). They are inducible, secreted and for the most part pro-inflammatory in nature, although certain chemokines are considered to be "homeostatic" concerned with the migration of cells during tissue development, maintenance or repair (Kehrl, 2006). Chemokines can be divided into three sub-populations based upon the position of four key cysteine residues; these are the 'CXC' family, 'CC' family, and the 'C' family (Kehrl, 2006). Chemokine biology is further complicated by the fact that these molecules share ~20%-90% homology in their amino acid residues (Laing and Secombes, 2004). Chemokine receptors are classical GPCRs and again have ~50% homology among each subfamily and ~30% homology between subfamilies (Kehrl, 2006). This all leads to a redundancy in chemokine signalling with certain receptors able to respond to several different chemokines. This in turn makes studying individual chemokines not only difficult but also physiologically inaccurate. Therefore when attempting to decipher the outcome of chemokine release from a cell following a specific stimulation it is beneficial to take into account the entire range chemokines released. While chemokines are exclusively chemotactic molecules, cytokines play other roles such as regulation of fever, inflammation, and inhibition of viral replication (Gruol and Nelson, 1997; Witowski et al., 2004). IL-6 however is a potent mediator of inflammation which acts mainly by promoting Th17 cell differentiation while inhibiting the expansion of the anti-inflammatory Treg population (Kimura and Kishimoto, 2010). It is also worth noting that nearly all anti-inflammatory cytokines have at least some pro-inflammatory properties. The overall effect of any given cytokine is dependent on a number of variables; the cellular components on which it acts, the expression level of its cognate receptor, the presence of competing or synergistic molecules, and the presence of inhibitory intracellular signals. We show here that the release of both cytokines (IL-6) and chemokine (LIX and RANTES) can be attenuated by S1P receptor modulation.

Much of our work has centred around astrocytes and S1P receptors expressed on these cell types. We show that astrocytes are capable of producing and releasing a wide range of cytokines and chemokines in response to both bacterial endotoxins such as LPS (**Fig.** 5.3) and cytokine stimulation such as IL-17 and TNF-α (**Fig.** 5.6). We also show that astrocyte expressed S1P receptors can be targeted to have an inhibitory effect on this release of pro-inflammatory cytokines. Future use of new specific S1P receptor antagonists should aid in understanding the exact role of these receptors in the inhibition of cytokine production. We believe that astrocytes play a key role in neuroinflammation by responding to various pro-inflammatory signals, in particular, the signature

cytokine of the pro-inflammatory Th17 cells. We propose that astrocytes respond to this activation by producing and releasing a large number of chemotactic molecules which results in a second wave of leukocyte infiltration into the CNS, which in turn propagates and exacerbates the inflammatory event (**Fig.** 5.11). Astrocytes are in a unique position to do this considering the role they play in maintaining the integrity of the BBB, and also their involvement with, and close proximity to both oligodendrocytes and neurons. Astrocytes are neither pro-inflammatory nor anti-inflammatory by nature; they are merely reactive to their surroundings. Depending on the inflammatory environment astrocytes can promote a Th1, Th2 or Th17 mediated response. Astrocytes maintain close gap junction mediated connectivity, this combined with astrocyte's morphological attributes means these cells can be both targets of, and responders to inflammation and infectious agents with wide reaching consequences (Eugenin et al., 2012; Kielian, 2008). Astrocytes are also in direct contact with the cerebral vasculature and the glial limitans, this allows them to couple expression of pro-inflammatory cytokines and chemokines with leukocyte infiltration of the CNS compartment (Paolinelli et al., 2011). The number of astrocytes, their ability to respond to and release pro-inflammatory mediators, their access to circulating blood and their key role in the BBB make them perfect candidates as drivers of neuroinflammation(Dong and Benveniste, 2001). This hypothesis is mirrored by observations that astrocytes are crucial in converting localised T-cell-APC interactions into a full blown inflammatory event characterised by leukocyte invasion of the CNS (Kang et al., 2010). The role of astrocytes in the milieu of interacting resident cells and infiltrates during the inflammatory process of active MS is not very well understood. While key players in this complex process have emerged over the years, how each of these cytokines, chemokines and signalling molecules contribute to the overall initiation and progression of neuroinflammation remains unclear.

6.1.5 Closing Remarks.

Gilenya® (FTY720-P) has recently received U.S. regulatory approval by the FDA and becomes the first drug in its class as an oral immunomodulatory drug for the treatment of RRMS. This oral therapy will no doubt supersede all current injection and I.V infusion therapies for the treatment of RRMS. The emergence of Gilenya® has exciting implications for patients diagnosed with MS and will provide these patients with improved disease management as well as an improved standard of living. While Gilenya® has proven itself to be more efficient at treating MS compared to current therapies, the long term safety of this oral therapy remains to established (Cohen et al., 2010; Yeh and Weinstock-Guttman, 2011). The ubiquitous expression of the S1P receptors on varied cell-types throughout the

body, coupled with the lipophilic nature of FTY720-P means the drug has a wide distribution profile, while reflects some concern. In trials the most common side-effects were; elevated liver enzymes, macular oedema, high blood pressure, shortness of breath, bronchitis, diarrhoea and bradycardia (Kappos et al., 2010). Two cases of fatal herpes infection resulting in progressive multifocal leukoencephalopathy occurred in trials using a dosage 1.25mg of Gilenya®, which is however 2.5 times the dose approved by the FDA. Since being approved for the treatment of RRMS reports have indicated a number of fatalities in patients treated with Gilenya®. Both the FDA and EMA (European Medicines Agency) have begun reviews of the medication following reports of 11 deaths from a patient size of approximately 33,000 (6 patients died of unknown causes and 5 from varying cardiac events) however these deaths have yet to be directly linked with Gilenya®. However screening of patienst for pre-existing cardiac abnormalities, coupled with an adequate observation period immediately after drug administration should reduce adverse effects. The emergence of Gilenya® as the first orally available first-line therapy for the treatment of MS is in itself a great achievement and is one that will benefit MS patients for years to come.

The peripheral immunological effects of FTY720-P have been well characterised, however protective modalities within the CNS have recently come to light (Choi et al., 2011; Gonzalez-Cabrera et al., 2012). Modulation of CNS expressed S1P receptor signalling pathways could potentially provide neuroprotection while bypassing the need for global immunosuppression. If functional CNS target(s) for FTY720-P can be elucidated and a specific CNS targeted therapy designed it would reduce the observed peripheral side-effects of Gilenya®. Considering the expression profile of S1P receptors it will become increasingly necessary to establish the effect FTY720-P has on different cell types. Here we study the drugs effect on astrocytes. In this thesis we provide compelling evidence that FTY720-P binds, activates, internalises and functionally antagonises S1P1 receptors in astrocytes in a pathway specific manner, and actively inhibits the release of pro-inflammatory cytokines from these cells. When taken together with other current research it places astrocytic S1P1 receptors central to explaining the CNS based effects of Gilenya®. Future studies aimed at characterising and modulating the cytokine/chemokine networks and defining the involvement of S1P receptors in the neuroinflammatory brain may open novel therapeutic windows both for the treatment of MS but also other diseases such as rheumatoid arthritis, systemic lupus erythematosus, asthma and psoriasis, in which inflammation plays a central role.

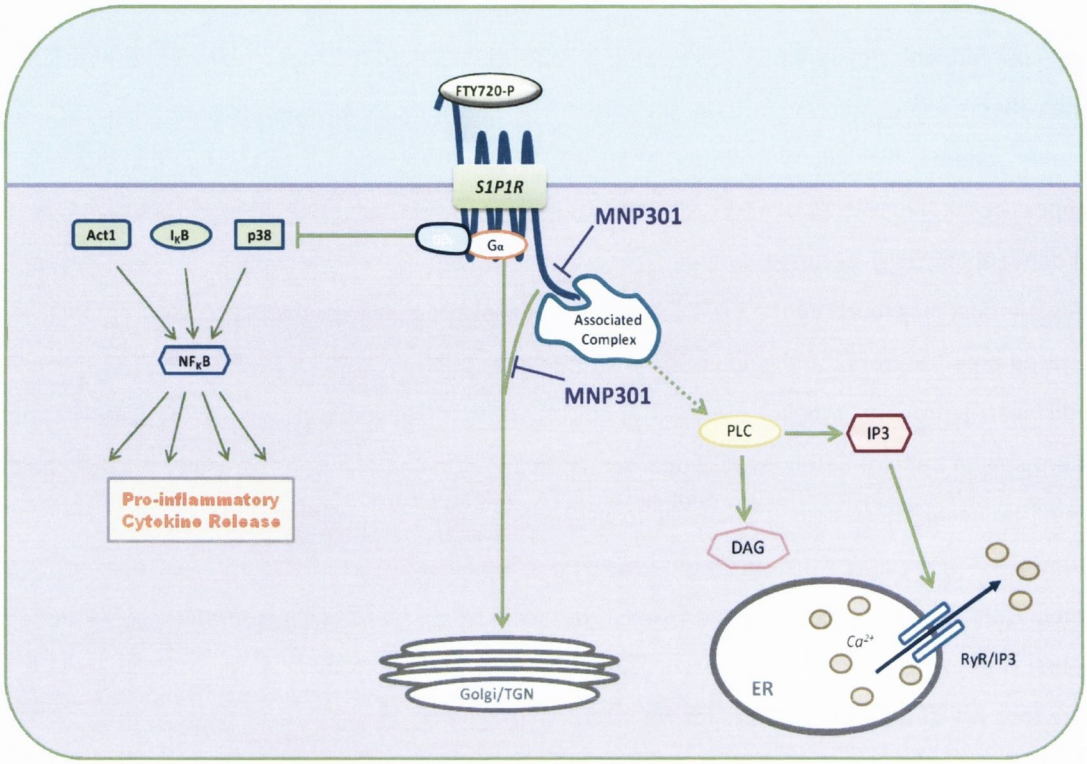


Figure 6.1 *Regulation of S1P1 Receptor Signalling and Trafficking in Astrocytes.*

In summary, we show that FTY720-P induced internalisation of the S1P1 receptor in astrocytes can be modulated by competivley inhibiting putative protein-protein interactions at the extreme c-terminus of the S1P1 receptor. In addition, we show that MNP301 does not exert an inhibitory effect on G-protein mediated inhibition of cAMP levels, however this peptide does specifically inhibit S1P1 receptor mediated Ca^{2+} signalling. Moreover, we show that FTY720-P pre-treatment can inhibit the release of a number of pro-inflammatory and chemotactic cytokines from both primary rat and human astrocytes. In this way we propose that FTY720-P can act on S1P receptors in the CNS to the release of molecules that can contribute to MS pathogenesis.

Chapter 7. References.

Adachi, K., Chiba, K., 2008. FTY720 story. Its discovery and the following accelerated development of sphingosine 1-phosphate receptor agonists as immunomodulators based on reverse pharmacology. Perspect Medicin Chem 1, 11-23.

Airas, L., Niemela, J., Yegutkin, G., Jalkanen, S., 2007. Mechanism of action of IFN-beta in the treatment of multiple sclerosis: a special reference to CD73 and adenosine. Ann N Y Acad Sci 1110, 641-648.

Albert, R., Hinterding, K., Brinkmann, V., Guerini, D., Muller-Hartwieg, C., Knecht, H., Simeon, C., Streiff, M., Wagner, T., Welzenbach, K., Zecri, F., Zollinger, M., Cooke, N., Francotte, E., 2005. Novel immunomodulator FTY720 is phosphorylated in rats and humans to form a single stereoisomer. Identification, chemical proof, and biological characterization of the biologically active species and its enantiomer. J Med Chem 48, 5373-5377.

Allen, N. J., Barres, B. A., 2005. Signaling between glia and neurons: focus on synaptic plasticity. Curr Opin Neurobiol 15, 542-548.

Allende, M. L., Dreier, J. L., Mandala, S., Proia, R. L., 2004. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. J Biol Chem 279, 15396-15401.

Almolda, B., Gonzalez, B., Castellano, B., 2011. Antigen presentation in EAE: role of microglia, macrophages and dendritic cells. Front Biosci 16, 1157-1171.

Antel, J. P., Miron, V. E., 2008. Central nervous system effects of current and emerging multiple sclerosis-directed immuno-therapies. Clin Neurol Neurosurg 110, 951-957.

Bacci, A., Verderio, C., Pravettoni, E., Matteoli, M., 1999. The role of glial cells in synaptic function. Philos Trans R Soc Lond B Biol Sci 354, 403-409.

Bakhiet, M., Tjernlund, A., Mousa, A., Gad, A., Stromblad, S., Kuziel, W. A., Seiger, A., Andersson, J., 2001. RANTES promotes growth and survival of human first-trimester forebrain astrocytes. Nat Cell Biol 3, 150-157.

Bal-Price, A., Brown, G. C., 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 21, 6480-6491.

Ballabh, P., Braun, A., Nedergaard, M., 2004. The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 16, 1-13.

Banks, W. A., Kastin, A. J., Broadwell, R. D., 1995. Passage of cytokines across the blood-brain barrier. Neuroimmunomodulation 2, 241-248.

Barres, B. A., Smith, S. J., 2001. Neurobiology. Cholesterol--making or breaking the synapse. Science 294, 1296-1297.

Bassi, R., Anelli, V., Giussani, P., Tettamanti, G., Viani, P., Riboni, L., 2006. Sphingosine-1-phosphate is released by cerebellar astrocytes in response to bFGF and induces astrocyte proliferation through G(i)-protein-coupled receptors. Glia 53, 621-630.

Ben Achour, S., Pont-Lezica, L., Bechade, C., Pascual, O., 2010. Is astrocyte calcium signaling relevant for synaptic plasticity? Neuron Glia Biol, 1-9.

Bettelli, E., Sullivan, B., Szabo, S. J., Sobel, R. A., Glimcher, L. H., Kuchroo, V. K., 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. J Exp Med 200, 79-87.

Bezprozvanny, I., Watras, J., Ehrlich, B. E., 1991. Bell-shaped calcium-response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature 351, 751-754.

Bielekova, B., Howard, T., Packer, A. N., Richert, N., Blevins, G., Ohayon, J., Waldmann, T. A., McFarland, H. F., Martin, R., 2009. Effect of anti-CD25 antibody daclizumab in the inhibition of inflammation and stabilization of disease progression in multiple sclerosis. Arch Neurol 66, 483-489.

Billich, A., Bornancin, F., Devay, P., Mechtcheriakova, D., Urtz, N., Baumruker, T., 2003. Phosphorylation of the immunomodulatory drug FTY720 by

sphingosine kinases. J Biol Chem 278, 47408-47415.
Birgbauer, E., Rao, T. S., Webb, M., 2004. Lysolecithin induces demyelination in vitro in a cerebellar slice culture system. J Neurosci Res 78, 157-166.
Birnbaumer, L., 1992. Receptor-to-effector signaling through G proteins: roles for beta gamma dimers as well as alpha subunits. Cell 71, 1069-1072.
Blakemore, W. F., 2008. Regeneration and repair in multiple sclerosis: the view of experimental pathology. J Neurol Sci 265, 1-4.
Bloomgren, G., Richman, S., Hotermans, C., Subramanyam, M., Goelz, S., Natarajan, A., Lee, S., Plavina, T., Scanlon, J. V., Sandrock, A., Bozic, C., 2012. Risk of natalizumab-associated progressive multifocal leukoencephalopathy. N Engl J Med 366, 1870-1880.
Boutin, A., Allen, M. D., Geras-Raaka, E., Huang, W., Neumann, S., Gershengorn, M. C., 2011. Thyrotropin receptor stimulates internalization-independent persistent phosphoinositide signaling. Mol Pharmacol 80, 240-246.
Brady, A. E., Limbird, L. E., 2002. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. Cell Signal 14, 297-309.
Brambilla, R., Bracchi-Ricard, V., Hu, W. H., Frydel, B., Bramwell, A., Karmally, S., Green, E. J., Bethea, J. R., 2005. Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. J Exp Med 202, 145-156.
Brinkmann, V., 2007. Sphingosine 1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. Pharmacol Ther 115, 84-105.
Brinkmann, V., 2009. FTY720 (fingolimod) in Multiple Sclerosis: therapeutic effects in the immune and the central nervous system. Br J Pharmacol 158, 1173-1182.
Brinkmann, V., Cyster, J. G., Hla, T., 2004. FTY720: sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. Am J Transplant 4, 1019-1025.
Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C. A., Zollinger, M., Lynch, K. R., 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem 277, 21453-21457.
Brousil, J. A., Roberts, R. J., Schlein, A. L., 2006. Cladribine: an investigational immunomodulatory agent for multiple sclerosis. Ann Pharmacother 40, 1814-1821.
Brown, B. A., 2009. Natalizumab in the treatment of multiple sclerosis. Ther Clin Risk Manag 5, 585-594.
Bruck, W., 2005. The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. J Neurol 252 Suppl 5, v3-9.
Bruck, W., Kuhlmann, T., Stadelmann, C., 2003. Remyelination in multiple sclerosis. J Neurol Sci 206, 181-185.
Calebiro, D., Nikolaev, V. O., Gagliani, M. C., de Filippis, T., Dees, C., Tacchetti, C., Persani, L., Lohse, M. J., 2009. Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. PLoS Biol 7, e1000172.
Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., von Zastrow, M., 1999. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. Nature 401, 286-290.
Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., Williamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A 72, 3666-3670.
Chabaud, M., Garnero, P., Dayer, J. M., Guerne, P. A., Fossiez, F., Miossec, P., 2000. Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis. Cytokine 12, 1092-1099.
Chae, S. S., Proia, R. L., Hla, T., 2004. Constitutive expression of the S1P1 receptor in adult tissues. Prostaglandins Other Lipid Mediat 73, 141-150.
Chang, M. S., McNinch, J., Basu, R., Simonet, S., 1994. Cloning and characterization of the human neutrophil-activating peptide (ENA-78) gene. J Biol Chem 269, 25277-25282.

Charles, A. C., Merrill, J. E., Dirksen, E. R., Sanderson, M. J., 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. Neuron 6, 983-992.

Charo, I. F., Ransohoff, R. M., 2006. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 354, 610-621.

Chastain, E. M., Miller, S. D., 2012. Molecular mimicry as an inducing trigger for CNS autoimmune demyelinating disease. Immunol Rev 245, 227-238.

Chaudhuri, A., 2012. Ocrelizumab in multiple sclerosis: risks and benefits. Lancet 379, 1196-1197; author reply 1197.

Chiba, K., 2005. FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. Pharmacol Ther 108, 308-319.

Cho, K., Francis, J. C., Hirbec, H., Dev, K., Brown, M. W., Henley, J. M., Bashir, Z. I., 2003. Regulation of kainate receptors by protein kinase C and metabotropic glutamate receptors. J Physiol 548, 723-730.

Choi, J. W., Gardell, S. E., Herr, D. R., Rivera, R., Lee, C. W., Noguchi, K., Teo, S. T., Yung, Y. C., Lu, M., Kennedy, G., Chun, J., 2011. FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. Proc Natl Acad Sci U S A 108, 751-756.

Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S., Tigyi, G., 2002. Lysophospholipid receptor nomenclature. Pharmacol Rev 54, 265-269.

Chun, J., Hartung, H. P., 2010. Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. Clin Neuropharmacol 33, 91-101.

Coelho, R. P., Payne, S. G., Bittman, R., Spiegel, S., Sato-Bigbee, C., 2007. The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. J Pharmacol Exp Ther 323, 626-635.

Cohen, J. A., Barkhof, F., Comi, G., Hartung, H. P., Khatri, B. O., Montalban, X., Pelletier, J., Capra, R., Gallo, P., Izquierdo, G., Tiel-Wilck, K., de Vera, A., Jin, J., Stites, T., Wu, S., Aradhye, S., Kappos, L., 2010. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med 362, 402-415.

Coles, A. J., Fox, E., Vladic, A., Gazda, S. K., Brinar, V., Selmaj, K. W., Skoromets, A., Stolyarov, I., Bass, A., Sullivan, H., Margolin, D. H., Lake, S. L., Moran, S., Palmer, J., Smith, M. S., Compston, D. A., 2012. Alemtuzumab more effective than interferon beta-1a at 5-year follow-up of CAMMS223 Clinical Trial. Neurology 78, 1069-1078.

Compston, A., Coles, A., 2002. Multiple sclerosis. Lancet 359, 1221-1231.

Confavreux, C., Vukusic, S., Moreau, T., Adeleine, P., 2000. Relapses and progression of disability in multiple sclerosis. N Engl J Med 343, 1430-1438.

Correale, J., Villa, A., 2007. The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting. Autoimmunity 40, 148-160.

Correale, J., Villa, A., 2009. Cellular Elements of the Blood-Brain Barrier. Neurochem Res.

Coursol, S., Le Stunff, H., Lynch, D. V., Gilroy, S., Assmann, S. M., Spiegel, S., 2005. Arabidopsis sphingosine kinase and the effects of phytosphingosine-1-phosphate on stomatal aperture. Plant Physiol 137, 724-737.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A., Sedgwick, J. D., 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421, 744-748.

Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., Spiegel, S., 1996. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature 381, 800-803.

Das Sarma, J., Ciric, B., Marek, R., Sadhukhan, S., Caruso, M. L., Shafagh, J., Fitzgerald, D. C., Shindler, K. S., Rostami, A., 2009. Functional interleukin-17 receptor A is expressed in central nervous system glia and upregulated in experimental autoimmune encephalomyelitis. J Neuroinflammation 6, 14.

Davis, R. J., 1999. Signal transduction by the c-Jun N-terminal kinase. Biochem Soc Symp 64, 1-12.
De Bock, M., Wang, N., Bol, M., Decrock, E., Ponsaerts, R., Bultynck, G., Dupont, G., Leybaert, L., 2012. Connexin 43 Hemichannels Contribute to Cytoplasmic Ca2+ Oscillations by Providing a Bimodal Ca2+-dependent Ca2+ Entry Pathway. J Biol Chem 287, 12250-12266.
de Vos, A. F., van Meurs, M., Brok, H. P., Boven, L. A., Hintzen, R. Q., van der Valk, P., Ravid, R., Rensing, S., Boon, L., t Hart, B. A., Laman, J. D., 2002. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. J Immunol 169, 5415-5423.
Dermietzel, R., Hertberg, E. L., Kessler, J. A., Spray, D. C., 1991. Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J Neurosci 11, 1421-1432.
Dev, K. K., 2007. PDZ domain protein-protein interactions: a case study with PICK1. Curr Top Med Chem 7, 3-20.
Dev, K. K., Mullershausen, F., Mattes, H., Kuhn, R. R., Bilbe, G., Hoyer, D., Mir, A., 2008. Brain sphingosine-1-phosphate receptors: implication for FTY720 in the treatment of multiple sclerosis. Pharmacol Ther 117, 77-93.
Dev, K. K., Nakajima, Y., Kitano, J., Braithwaite, S. P., Henley, J. M., Nakanishi, S., 2000. PICK1 interacts with and regulates PKC phosphorylation of mGLUR7. J Neurosci 20, 7252-7257.
Dev, K. K., Nishimune, A., Henley, J. M., Nakanishi, S., 1999. The protein kinase C alpha binding protein PICK1 interacts with short but not long form alternative splice variants of AMPA receptor subunits. Neuropharmacology 38, 635-644.
Dong, Y., Benveniste, E. N., 2001. Immune function of astrocytes. Glia 36, 180-190.
Dorf, M. E., Berman, M. A., Tanabe, S., Heesen, M., Luo, Y., 2000. Astrocytes express functional chemokine receptors. J Neuroimmunol 111, 109-121.
Durelli, L., Conti, L., Clerico, M., Boselli, D., Contessa, G., Ripellino, P., Ferrero, B., Eid, P., Novelli, F., 2009. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. Ann Neurol 65, 499-509.
English, D., Welch, Z., Kovala, A. T., Harvey, K., Volpert, O. V., Brindley, D. N., Garcia, J. G., 2000. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J 14, 2255-2265.
Eskan, M. A., Rose, B. G., Benakanakere, M. R., Zeng, Q., Fujioka, D., Martin, M. H., Lee, M. J., Kinane, D. F., 2008. TLR4 and S1P receptors cooperate to enhance inflammatory cytokine production in human gingival epithelial cells. Eur J Immunol 38, 1138-1147.
Eugenin, E. A., Basilio, D., Saez, J. C., Orellana, J. A., Raine, C. S., Bukauskas, F., Bennett, M. V., Berman, J. W., 2012. The Role of Gap Junction Channels During Physiologic and Pathologic Conditions of the Human Central Nervous System. J Neuroimmune Pharmacol.
Farina, C., Aloisi, F., Meinl, E., 2007. Astrocytes are active players in cerebral innate immunity. Trends Immunol 28, 138-145.
Ferber, I. A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D., Fathman, C. G., 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J Immunol 156, 5-7.
Fiacco, T. A., McCarthy, K. D., 2006. Astrocyte calcium elevations: properties, propagation, and effects on brain signaling. Glia 54, 676-690.
Fischer, I., Alliod, C., Martinier, N., Newcombe, J., Brana, C., Pouly, S., 2011. Sphingosine kinase 1 and sphingosine 1-phosphate receptor 3 are functionally upregulated on astrocytes under pro-inflammatory conditions. PLoS One 6, e23905.
Flier, J., Boorsma, D. M., van Beek, P. J., Nieboer, C., Stoof, T. J., Willemze, R., Tensen, C. P., 2001. Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. J Pathol 194, 398-405.
Foster, C. A., Howard, L. M., Schweitzer, A., Persohn, E., Hiestand, P. C., Balatoni, B.,

Reuschel, R., Beerli, C., Schwartz, M., Billich, A., 2007. Brain penetration of the oral immunomodulatory drug FTY720 and its phosphorylation in the central nervous system during experimental autoimmune encephalomyelitis: consequences for mode of action in multiple sclerosis. J Pharmacol Exp Ther 323, 469-475.

Fox, E. J., 2006. Management of worsening multiple sclerosis with mitoxantrone: a review. Clin Ther 28, 461-474.

Franke, H., Verkhratsky, A., Burnstock, G., Illes, P., 2012. Pathophysiology of astroglial purinergic signalling. Purinergic Signal 8, 629-657.

Franklin, R. J., Ffrench-Constant, C., 2008. Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci 9, 839-855.

Frei, K., Malipiero, U. V., Leist, T. P., Zinkernagel, R. M., Schwab, M. E., Fontana, A., 1989. On the cellular source and function of interleukin 6 produced in the central nervous system in viral diseases. Eur J Immunol 19, 689-694.

Frohman, E. M., Filippi, M., Stuve, O., Waxman, S. G., Corboy, J., Phillips, J. T., Lucchinetti, C., Wilken, J., Karandikar, N., Hemmer, B., Monson, N., De Keyser, J., Hartung, H., Steinman, L., Oksenberg, J. R., Cree, B. A., Hauser, S., Racke, M. K., 2005. Characterizing the mechanisms of progression in multiple sclerosis: evidence and new hypotheses for future directions. Arch Neurol 62, 1345-1356.

Furtado, G. C., Pina, B., Tacke, F., Gaupp, S., van Rooijen, N., Moran, T. M., Randolph, G. J., Ransohoff, R. M., Chensue, S. W., Raine, C. S., Lira, S. A., 2006. A novel model of demyelinating encephalomyelitis induced by monocytes and dendritic cells. J Immunol 177, 6871-6879.

Furukawa, A., Kita, K., Toyomoto, M., Fujii, S., Inoue, S., Hayashi, K., Ikeda, K., 2007. Production of nerve growth factor enhanced in cultured mouse astrocytes by glycerophospholipids, sphingolipids, and their related compounds. Mol Cell Biochem 305, 27-34.

Garcia-Regalado, A., Guzman-Hernandez, M. L., Ramirez-Rangel, I., Robles-Molina, E., Balla, T., Vazquez-Prado, J., Reyes-Cruz, G., 2008. G protein-coupled receptor-promoted trafficking of Gbeta1gamma2 leads to AKT activation at endosomes via a mechanism mediated by Gbeta1gamma2-Rab11a interaction. Mol Biol Cell 19, 4188-4200.

Ghosh, T. K., Bian, J., Gill, D. L., 1994. Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored calcium. J Biol Chem 269, 22628-22635.

Giaume, C., Koulakoff, A., Roux, L., Holcman, D., Rouach, N., 2010. Astroglial networks: a step further in neuroglial and gliovascular interactions. Nat Rev Neurosci 11, 87-99.

Giussani, P., Ferraretto, A., Gravaghi, C., Bassi, R., Tettamanti, G., Riboni, L., Viani, P., 2007. Sphingosine-1-phosphate and calcium signaling in cerebellar astrocytes and differentiated granule cells. Neurochem Res 32, 27-37.

Goetzl, E. J., Graler, M. H., 2004. Sphingosine 1-phosphate and its type 1 G protein-coupled receptor: trophic support and functional regulation of T lymphocytes. J Leukoc Biol 76, 30-35.

Golovina, V. A., 2005. Visualization of localized store-operated calcium entry in mouse astrocytes. Close proximity to the endoplasmic reticulum. J Physiol 564, 737-749.

Gonzalez-Cabrera, P. J., Cahalan, S. M., Nguyen, N., Sarkisyan, G., Leaf, N. B., Cameron, M. D., Kago, T., Rosen, H., 2012. S1P1 Receptor Modulation with Cyclical Recovery from Lymphopenia Ameliorates Mouse Model of Multiple Sclerosis. Mol Pharmacol 81, 166-174.

Gonzalez-Cabrera, P. J., Jo, E., Sanna, M. G., Brown, S., Leaf, N., Marsolais, D., Schaeffer, M. T., Chapman, J., Cameron, M., Guerrero, M., Roberts, E., Rosen, H., 2008. Full Pharmacological Efficacy of a Novel S1P(1) Agonist That Does Not Require S1P-Like Headgroup Interactions. Molecular Pharmacology 74, 1308-1318.

Gorospe, J. R., Maletkovic, J., 2006. Alexander disease and megalencephalic leukoencephalopathy with subcortical cysts: leukodystrophies arising from astrocyte

dysfunction. Ment Retard Dev Disabil Res Rev 12, 113-122.
Graber, J. J., Allie, S. R., Mullen, K. M., Jones, M. V., Wang, T., Krishnan, C., Kaplin, A. I., Nath, A., Kerr, D. A., Calabresi, P. A., 2008. Interleukin-17 in transverse myelitis and multiple sclerosis. J Neuroimmunol 196, 124-132.
Gregory, S. G., Schmidt, S., Seth, P., Oksenberg, J. R., Hart, J., Prokop, A., Caillier, S. J., Ban, M., Goris, A., Barcellos, L. F., Lincoln, R., McCauley, J. L., Sawcer, S. J., Compston, D. A., Dubois, B., Hauser, S. L., Garcia-Blanco, M. A., Pericak-Vance, M. A., Haines, J. L., 2007. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. Nat Genet 39, 1083-1091.
Group, T. L. M. S. S., 1999. TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. Neurology 53, 457-465.
Gruol, D. L., Nelson, T. E., 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Mol Neurobiol 15, 307-339.
Haegeman, G., Content, J., Volckaert, G., Derynck, R., Tavernier, J., Fiers, W., 1986. Structural analysis of the sequence coding for an inducible 26-kDa protein in human fibroblasts. Eur J Biochem 159, 625-632.
Hafler, D. A., Compston, A., Sawcer, S., Lander, E. S., Daly, M. J., De Jager, P. L., de Bakker, P. I., Gabriel, S. B., Mirel, D. B., Ivinson, A. J., Pericak-Vance, M. A., Gregory, S. G., Rioux, J. D., McCauley, J. L., Haines, J. L., Barcellos, L. F., Cree, B., Oksenberg, J. R., Hauser, S. L., 2007. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 357, 851-862.
Hagemeier, K., Bruck, W., Kuhlmann, T., 2012. Multiple sclerosis - remyelination failure as a cause of disease progression. Histol Histopathol 27, 277-287.
Hait, N. C., Oskeritzian, C. A., Paugh, S. W., Milstien, S., Spiegel, S., 2006. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. Biochim Biophys Acta 1758, 2016-2026.
Halassa, M. M., Haydon, P. G., 2010. Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. Annu Rev Physiol 72, 335-355.
Hanson, M. A., Roth, C. B., Jo, E., Griffith, M. T., Scott, F. L., Reinhart, G., Desale, H., Clemons, B., Cahalan, S. M., Schuerer, S. C., Sanna, M. G., Han, G. W., Kuhn, P., Rosen, H., Stevens, R. C., 2012. Crystal structure of a lipid G protein-coupled receptor. Science 335, 851-855.
Hanyaloglu, A. C., von Zastrow, M., 2008. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu Rev Pharmacol Toxicol 48, 537-568.
Hasegawa, Y., Suzuki, H., Sozen, T., Rolland, W., Zhang, J. H., 2009. Activation of Sphingosine 1-Phosphate Receptor-1 by FTY720 Is Neuroprotective After Ischemic Stroke in Rats. Stroke.
Hatton, G. I., 2002. Glial-neuronal interactions in the mammalian brain. Adv Physiol Educ 26, 225-237.
He, F., Sun, Y. E., 2007. Glial cells more than support cells? Int J Biochem Cell Biol 39, 661-665.
He, X., Huang, Y., Li, B., Gong, C. X., Schuchman, E. H., 2008. Deregulation of sphingolipid metabolism in Alzheimer's disease. Neurobiol Aging 31, 398-408.
Hellings, N., Baree, M., Verhoeven, C., D'Hooghe M, B., Medaer, R., Bernard, C. C., Raus, J., Stinissen, P., 2001. T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls. J Neurosci Res 63, 290-302.
Henderson, A. P., Barnett, M. H., Parratt, J. D., Prineas, J. W., 2009. Multiple sclerosis: distribution of inflammatory cells in newly forming lesions. Ann Neurol 66, 739-753.
Hendriks, J. J., Teunissen, C. E., de Vries, H. E., Dijkstra, C. D., 2005. Macrophages and neurodegeneration. Brain Res Brain Res Rev 48, 185-195.
Henning, G., Ohl, L., Junt, T., Reiterer, P., Brinkmann, V., Nakano, H., Hohenberger, W., Lipp, M., Forster, R., 2001. CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. J Exp Med 194, 1875-1881.

Hildebrand, P. W., Scheerer, P., Park, J. H., Choe, H. W., Piechnick, R., Ernst, O. P., Hofmann, K. P., Heck, M., 2009. A ligand channel through the G protein coupled receptor opsin. PLoS One 4, e4382.

Hill, K. E., Zollinger, L. V., Watt, H. E., Carlson, N. G., Rose, J. W., 2004. Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. J Neuroimmunol 151, 171-179.

Hla, T., Brinkmann, V., 2012. Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation. Neurology 76, S3-8.

Hla, T., Venkataraman, K., Michaud, J., 2008. The vascular S1P gradient-cellular sources and biological significance. Biochim Biophys Acta 1781, 477-482.

Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S., Spiegel, S., 2001. Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. Science 291, 1800-1803.

Hofstetter, H. H., Ibrahim, S. M., Koczan, D., Kruse, N., Weishaupt, A., Toyka, K. V., Gold, R., 2005. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. Cell Immunol 237, 123-130.

Hopson, K. P., Truelove, J., Chun, J., Wang, Y., Waeber, C., 2011. S1P activates store-operated calcium entry via receptor- and non-receptor-mediated pathways in vascular smooth muscle cells. Am J Physiol Cell Physiol 300, C919-926.

Horga, A., Castillo, J., Montalban, X., 2010. Fingolimod for relapsing multiple sclerosis: an update. Expert Opin Pharmacother 11, 1183-1196.

Hoth, M., Penner, R., 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355, 353-356.

Hu, Y., Lee, X., Ji, B., Guckian, K., Apicco, D., Pepinsky, R. B., Miller, R. H., Mi, S., 2011. Sphingosine 1-phosphate receptor modulator fingolimod (FTY720) does not promote remyelination in vivo. Mol Cell Neurosci 48, 72-81.

Hughes, B., 2009. First oral therapies for multiple sclerosis anticipated. Nat Rev Drug Discov 8, 831-833.

Huppert, J., Closhen, D., Croxford, A., White, R., Kulig, P., Pietrowski, E., Bechmann, I., Becher, B., Luhmann, H. J., Waisman, A., Kuhlmann, C. R., 2010. Cellular mechanisms of IL-17-induced blood-brain barrier disruption. FASEB J 24, 1023-1034.

Hutchinson, M., 2009. Predicting and preventing the future: actively managing multiple sclerosis. Pract Neurol 9, 133-143, discussion 144.

Igarashi, J., Michel, T., 2000. Agonist-modulated targeting of the EDG-1 receptor to plasmalemmal caveolae. eNOS activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. J Biol Chem 275, 32363-32370.

Im, D. S., Heise, C. E., Ancellin, N., O'Dowd, B. F., Shei, G. J., Heavens, R. P., Rigby, M. R., Hla, T., Mandala, S., McAllister, G., George, S. R., Lynch, K. R., 2000. Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. J Biol Chem 275, 14281-14286.

Imasawa, T., Koike, K., Ishii, I., Chun, J., Yatomi, Y., 2010. Blockade of sphingosine 1-phosphate receptor 2 signaling attenuates streptozotocin-induced apoptosis of pancreatic beta-cells. Biochem Biophys Res Commun 392, 207-211.

Ishibashi, T., Dakin, K. A., Stevens, B., Lee, P. R., Kozlov, S. V., Stewart, C. L., Fields, R. D., 2006. Astrocytes promote myelination in response to electrical impulses. Neuron 49, 823-832.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y., 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236, 313-322.

Jackson, S. J., Giovannoni, G., Baker, D., 2011. Fingolimod modulates microglial activation to augment markers of remyelination. J Neuroinflammation 8, 76.

Jadidi-Niaragh, F., Mirshafiey, A., 2011. Th17 cell, the new player of neuroinflammatory

process in multiple sclerosis. Scand J Immunol 74, 1-13.
Jaillard, C., Harrison, S., Stankoff, B., Aigrot, M. S., Calver, A. R., Duddy, G., Walsh, F. S., Pangalos, M. N., Arimura, N., Kaibuchi, K., Zalc, B., Lubetzki, C., 2005. Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. J Neurosci 25, 1459-1469.
Jenkins, G. M., Hannun, Y. A., 2001. Role for de novo sphingoid base biosynthesis in the heat-induced transient cell cycle arrest of Saccharomyces cerevisiae. J Biol Chem 276, 8574-8581.
Jensen, J., Krakauer, M., Sellebjerg, F., 2005. Cytokines and adhesion molecules in multiple sclerosis patients treated with interferon-beta1b. Cytokine 29, 24-30.
Kang, Z., Altuntas, C. Z., Gulen, M. F., Liu, C., Giltiay, N., Qin, H., Liu, L., Qian, W., Ransohoff, R. M., Bergmann, C., Stohlman, S., Tuohy, V. K., Li, X., 2010. Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. Immunity 32, 414-425.
Kapitonov, D., Allegood, J. C., Mitchell, C., Hait, N. C., Almenara, J. A., Adams, J. K., Zipkin, R. E., Dent, P., Kordula, T., Milstien, S., Spiegel, S., 2009. Targeting sphingosine kinase 1 inhibits Akt signaling, induces apoptosis, and suppresses growth of human glioblastoma cells and xenografts. Cancer Res 69, 6915-6923.
Kappos, L., Gold, R., Miller, D. H., Macmanus, D. G., Havrdova, E., Limmroth, V., Polman, C. H., Schmierer, K., Yousry, T. A., Yang, M., Eraksoy, M., Meluzinova, E., Rektor, I., Dawson, K. T., Sandrock, A. W., O'Neill, G. N., 2008. Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study. Lancet 372, 1463-1472.
Kappos, M. D., Ernst-Wilhelm Radue, M.D., Paul O'Connor, M.D., Chris Polman, M.D.,, Reinhard Hohlfeld, M. D., Peter Calabresi, M.D., Krzysztof Selmaj, M.D., Catherine Agoropoulou, Ph.D.,, Malgorzata Leyk, P. D., Lixin Zhang-Auberson, M.D., Ph.D., and Pascale Burtin, M.D., Ph.D.,, Group*, f. t. F. S., 2010. A Placebo-Controlled Trial of Oral Fingolimod in Relapsing Multiple Sclerosis. The New England Journal of Medicine 362, 1-15.
Ke, Y., Jiang, G., Sun, D., Kaplan, H. J., Shao, H., 2009. Retinal Astrocytes respond to IL-17 differently than Retinal Pigment Epithelial cells. J Leukoc Biol 86, 1377-1384.
Kehrl, J. H., 2006. Chemoattractant receptor signaling and the control of lymphocyte migration. Immunol Res 34, 211-227.
Kero, J., Ahmed, K., Wettschureck, N., Tunaru, S., Wintermantel, T., Greiner, E., Schutz, G., Offermanns, S., 2007. Thyrocyte-specific Gq/G11 deficiency impairs thyroid function and prevents goiter development. J Clin Invest 117, 2399-2407.
Kielian, T., 2008. Glial connexins and gap junctions in CNS inflammation and disease. J Neurochem 106, 1000-1016.
Kim, H. J., Miron, V. E., Dukala, D., Proia, R. L., Ludwin, S. K., Traka, M., Antel, J. P., Soliven, B., 2011. Neurobiological effects of sphingosine 1-phosphate receptor modulation in the cuprizone model. FASEB J 25, 1509-1518.
Kimura, A., Kishimoto, T., 2010. IL-6: regulator of Treg/Th17 balance. Eur J Immunol 40, 1830-1835.
Kimura, A., Ohmori, T., Ohkawa, R., Madoiwa, S., Mimuro, J., Murakami, T., Kobayashi, E., Hoshino, Y., Yatomi, Y., Sakata, Y., 2007. Essential roles of sphingosine 1-phosphate/S1P1 receptor axis in the migration of neural stem cells toward a site of spinal cord injury. Stem Cells 25, 115-124.
Kinouchi, K., Brown, G., Pasternak, G., Donner, D. B., 1991. Identification and characterization of receptors for tumor necrosis factor-alpha in the brain. Biochem Biophys Res Commun 181, 1532-1538.
Korn, T., Magnus, T., Toyka, K., Jung, S., 2004. Modulation of effector cell functions in experimental autoimmune encephalomyelitis by leflunomide--mechanisms independent of pyrimidine depletion. J Leukoc Biol 76, 950-960.
Kreft, M., Stenovec, M., Rupnik, M., Grilc, S., Krzan, M., Potokar, M., Pangrsic, T., Haydon, P. G., Zorec, R., 2004. Properties of Ca(2+)-

dependent exocytosis in cultured astrocytes. Glia 46, 437-445.
Kroenke, M. A., Carlson, T. J., Andjelkovic, A. V., Segal, B. M., 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. J Exp Med 205, 1535-1541.
Kumar, R., Ferez, M., Swamy, M., Arechaga, I., Rejas, M. T., Valpuesta, J. M., Schamel, W. W., Alarcon, B., van Santen, H. M., 2011. Increased sensitivity of antigen-experienced T cells through the enrichment of oligomeric T cell receptor complexes. Immunity 35, 375-387.
Laing, K. J., Secombes, C. J., 2004. Chemokines. Dev Comp Immunol 28, 443-460.
Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., Cua, D. J., 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 201, 233-240.
Laugwitz, K. L., Allgeier, A., Offermanns, S., Spicher, K., Van Sande, J., Dumont, J. E., Schultz, G., 1996. The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. Proc Natl Acad Sci U S A 93, 116-120.
Lee, H., Deng, J., Kujawski, M., Yang, C., Liu, Y., Herrmann, A., Kortylewski, M., Horne, D., Somlo, G., Forman, S., Jove, R., Yu, H., 2010. STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. Nat Med 16, 1421-1428.
Lee, M. J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., Hla, T., 1998. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. Science 279, 1552-1555.
Li, R., Messing, A., Goldman, J. E., Brenner, M., 2002. GFAP mutations in Alexander disease. Int J Dev Neurosci 20, 259-268.
Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V., Wu, D., 2000. Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. Science 287, 1046-1049.
Li, Z., Zhang, Y., Sun, B., 2011. Current understanding of Th2 cell differentiation and function. Protein Cell 2, 604-611.
Linker, R. A., Kieseier, B. C., Gold, R., 2008. Identification and development of new therapeutics for multiple sclerosis. Trends Pharmacol Sci 29, 558-565.
Liu, B., Chen, H., Johns, T. G., Neufeld, A. H., 2006. Epidermal growth factor receptor activation: an upstream signal for transition of quiescent astrocytes into reactive astrocytes after neural injury. J Neurosci 26, 7532-7540.
Liu, B., Neufeld, A. H., 2007. Activation of epidermal growth factor receptors in astrocytes: from development to neural injury. J Neurosci Res 85, 3523-3529.
Liu, J., Marino, M. W., Wong, G., Grail, D., Dunn, A., Bettadapura, J., Slavin, A. J., Old, L., Bernard, C. C., 1998. TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. Nat Med 4, 78-83.
Liu, L., Belkadi, A., Darnall, L., Hu, T., Drescher, C., Cotleur, A. C., Padovani-Claudio, D., He, T., Choi, K., Lane, T. E., Miller, R. H., Ransohoff, R. M., 2010. CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. Nat Neurosci 13, 319-326.
Lo, C. G., Xu, Y., Proia, R. L., Cyster, J. G., 2005. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J Exp Med 201, 291-301.
Lo, K. J., Luk, H. N., Chin, T. Y., Chueh, S. H., 2002. Store depletion-induced calcium influx in rat cerebellar astrocytes. Br J Pharmacol 135, 1383-1392.
Lockhart, E., Green, A. M., Flynn, J. L., 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177, 4662-4669.
Locksley, R. M., Killeen, N., Lenardo, M. J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104, 487-501.
Lu, W., Maheshwari, A., Misiuta, I., Fox, S. E., Chen, N., Zigova, T., Christensen, R. D., Calhoun, D. A., 2005. Neutrophil-specific

chemokines are produced by astrocytic cells but not by neuronal cells. Brain Res Dev Brain Res 155, 127-134.

Lund, S., Christensen, K. V., Hedtjarn, M., Mortensen, A. L., Hagberg, H., Falsig, J., Hasseldam, H., Schrattenholz, A., Porzgen, P., Leist, M., 2006. The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. J Neuroimmunol 180, 71-87.

Luo, Y., Berman, M. A., Zhai, Q., Fischer, F. R., Abromson-Leeman, S. R., Zhang, Y., Kuziel, W. A., Gerard, C., Dorf, M. E., 2002. RANTES stimulates inflammatory cascades and receptor modulation in murine astrocytes. Glia 39, 19-30.

Luttrell, L. M., Lefkowitz, R. J., 2002. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115, 455-465.

Ma, X., Reynolds, S. L., Baker, B. J., Li, X., Benveniste, E. N., Qin, H., 2012. IL-17 enhancement of the IL-6 signaling cascade in astrocytes. J Immunol 184, 4898-4906.

Maceyka, M., Harikumar, K. B., Milstien, S., Spiegel, S., 2012. Sphingosine-1-phosphate signaling and its role in disease. Trends Cell Biol 22, 50-60.

Maeda, Y., Seki, N., Sato, N., Sugahara, K., Chiba, K., 2010. Sphingosine 1-phosphate receptor type 1 regulates egress of mature T cells from mouse bone marrow. Int Immunol 22, 515-525.

Maghazachi, A. A., Al-Aoukaty, A., Schall, T. J., 1996. CC chemokines induce the generation of killer cells from CD56+ cells. Eur J Immunol 26, 315-319.

Maier, L. M., Hafler, D. A., 2008. The developing mosaic of autoimmune disease risk. Nat Genet 40, 131-132.

Malchinkhuu, E., Sato, K., Muraki, T., Ishikawa, K., Kuwabara, A., Okajima, F., 2003. Assessment of the role of sphingosine 1-phosphate and its receptors in high-density lipoprotein-induced stimulation of astroglial cell function. Biochem J 370, 817-827.

Malek, R. L., Toman, R. E., Edsall, L. C., Wong, S., Chiu, J., Letterle, C. A., Van Brocklyn, J. R., Milstien, S., Spiegel, S., Lee, N. H., 2001. Nrg-1 belongs to the endothelial differentiation gene family of G protein-coupled sphingosine-1-phosphate receptors. J Biol Chem 276, 5692-5699.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., Rosen, H., 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 296, 346-349.

Marchese, A., Benovic, J. L., 2001. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. J Biol Chem 276, 45509-45512.

Marchese, A., Chen, C., Kim, Y. M., Benovic, J. L., 2003. The ins and outs of G protein-coupled receptor trafficking. Trends Biochem Sci 28, 369-376.

Marik, C., Felts, P. A., Bauer, J., Lassmann, H., Smith, K. J., 2007. Lesion genesis in a subset of patients with multiple sclerosis: a role for innate immunity? Brain 130, 2800-2815.

Martins, T. B., Rose, J. W., Jaskowski, T. D., Wilson, A. R., Husebye, D., Seraj, H. S., Hill, H. R., 2011. Analysis of proinflammatory and anti-inflammatory cytokine serum concentrations in patients with multiple sclerosis by using a multiplexed immunoassay. Am J Clin Pathol 136, 696-704.

Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L., Cyster, J. G., 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427, 355-360.

Matsuo, Y., Miura, S., Kawamura, A., Uehara, Y., Rye, K. A., Saku, K., 2007. Newly developed reconstituted high-density lipoprotein containing sphingosine-1-phosphate induces endothelial tube formation. Atherosclerosis 194, 159-168.

Matusevicius, D., Kivisakk, P., He, B., Kostulas, N., Ozenci, V., Fredrikson, S., Link, H., 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. Mult Scler 5, 101-104.

McCormack, P. L., Scott, L. J., 2004. Spotlight on Interferon-beta-1b in relapsing-remitting

and secondary progressive multiple sclerosis. BioDrugs 18, 343-347.
McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., Lefkowitz, R. J., 2000. Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science 290, 1574-1577.
McDonnell, G. V., Mawhinney, H., Graham, C. A., Hawkins, S. A., Middleton, D., 1999. A study of the HLA-DR region in clinical subgroups of multiple sclerosis and its influence on prognosis. J Neurol Sci 165, 77-83.
McQuaid, S., Cunnea, P., McMahon, J., Fitzgerald, U., 2009. The effects of blood-brain barrier disruption on glial cell function in multiple sclerosis. Biochem Soc Trans 37, 329-331.
Means, C. K., Miyamoto, S., Chun, J., Brown, J. H., 2008. S1P1 receptor localization confers selectivity for Gi-mediated cAMP and contractile responses. J Biol Chem 283, 11954-11963.
Meares, G. P., Ma, X., Qin, H., Benveniste, E. N., 2012. Regulation of CCL20 expression in astrocytes by IL-6 and IL-17. Glia 60, 771-781.
Meeuwsen, S., Persoon-Deen, C., Bsibsi, M., Ravid, R., van Noort, J. M., 2003. Cytokine, chemokine and growth factor gene profiling of cultured human astrocytes after exposure to proinflammatory stimuli. Glia 43, 243-253.
Mehling, M., Brinkmann, V., Antel, J., Bar-Or, A., Goebels, N., Vedrine, C., Kristofic, C., Kuhle, J., Lindberg, R. L. P., Kappos, L., 2008. FTY720 therapy exerts differential effects on T cell subsets in multiple sclerosis. Neurology 71, 1261-1267.
Mehling, M., Lindberg, R., Raulf, F., Kuhle, J., Hess, C., Kappos, L., Brinkmann, V., 2010. Th17 central memory T cells are reduced by FTY720 in patients with multiple sclerosis. Neurology 75, 403-410.
Miljkovic, D., Timotijevic, G., Mostarica Stojkovic, M., 2011. Astrocytes in the tempest of multiple sclerosis. FEBS Lett 585, 3781-3788.
Miller, M. D., Krangel, M. S., 1992. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. Crit Rev Immunol 12, 17-46.
Minagar, A., Alexander, J. S., 2003. Blood-brain barrier disruption in multiple sclerosis. Mult Scler 9, 540-549.
Miron, V. E., Jung, C. G., Kim, H. J., Kennedy, T. E., Soliven, B., Antel, J. P., 2008a. FTY720 modulates human oligodendrocyte progenitor process extension and survival. Ann Neurol 63, 61-71.
Miron, V. E., Schubart, A., Antel, J. P., 2008b. Central nervous system-directed effects of FTY720 (fingolimod). J Neurol Sci 274, 13-17.
Mitra, P., Oskeritzian, C. A., Payne, S. G., Beaven, M. A., Milstien, S., Spiegel, S., 2006. Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. Proc Natl Acad Sci U S A 103, 16394-16399.
Mullershausen, F., Craveiro, L. M., Shin, Y., Cortes-Cros, M., Bassilana, F., Osinde, M., Wisbart, W. L., Thallmair, M., Schwab, M. E., Sivasankaran, R., Seuwen, K., Dev, K. K., 2007. Phosphorylated FTY720 promotes astrocyte migration through sphingosine-1-phosphate receptors. Journal of Neurochemistry 102, 1151-1161.
Mullershausen, F., Zecri, F., Cetin, C., Billich, A., Guerini, D., Seuwen, K., 2009. Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. Nat Chem Biol 5, 428-434.
Murakami, M., Nishimoto, N., 2011. The value of blocking IL-6 outside of rheumatoid arthritis: current perspective. Curr Opin Rheumatol 23, 273-277.
Murata, N., Sato, K., Kon, J., Tomura, H., Okajima, F., 2000a. Quantitative measurement of sphingosine 1-phosphate by radioreceptor-binding assay. Anal Biochem 282, 115-120.
Murata, N., Sato, K., Kon, J., Tomura, H., Yanagita, M., Kuwabara, A., Ui, M., Okajima, F., 2000b. Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. Biochem J 352 Pt 3, 809-815.
Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D., Cua, D. J., 2003. Divergent pro- and antiinflammatory roles for

IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 198, 1951-1957.
Muscoli, C., Doyle, T., Dagostino, C., Bryant, L., Chen, Z., Watkins, L. R., Ryerse, J., Bieberich, E., Neumman, W., Salvemini, D., 2010. Counter-regulation of opioid analgesia by glial-derived bioactive sphingolipids. J Neurosci 30, 15400-15408.
Nair, A., Frederick, T. J., Miller, S. D., 2008. Astrocytes in multiple sclerosis: a product of their environment. Cell Mol Life Sci 65, 2702-2720.
Nash, B., Thomson, C. E., Linington, C., Arthur, A. T., McClure, J. D., McBride, M. W., Barnett, S. C., 2011. Functional duality of astrocytes in myelination. J Neurosci 31, 13028-13038.
Neuhaus, O., Farina, C., Wekerle, H., Hohlfeld, R., 2001. Mechanisms of action of glatiramer acetate in multiple sclerosis. Neurology 56, 702-708.
Neurath, M. F., Finotto, S., 2011. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. Cytokine Growth Factor Rev 22, 83-89.
Nichols, J. R., Aldrich, A. L., Mariani, M. M., Vidlak, D., Esen, N., Kielian, T., 2009. TLR2 deficiency leads to increased Th17 infiltrates in experimental brain abscesses. J Immunol 182, 7119-7130.
Nishimura, H., Akiyama, T., Irei, I., Hamazaki, S., Sadahira, Y., 2010. Cellular localization of sphingosine-1-phosphate receptor 1 expression in the human central nervous system. J Histochem Cytochem 58, 847-856.
Norimatsu, Y., Ohmori, T., Kimura, A., Madoiwa, S., Mimuro, J., Seichi, A., Yatomi, Y., Hoshino, Y., Sakata, Y., 2012. FTY720 improves functional recovery after spinal cord injury by primarily nonimmunomodulatory mechanisms. Am J Pathol 180, 1625-1635.
Noronha, A., Toscas, A., Jensen, M. A., 1993. Interferon beta decreases T cell activation and interferon gamma production in multiple sclerosis. J Neuroimmunol 46, 145-153.
Noseworthy, J. H., Lucchinetti, C., Rodriguez, M., Weinshenker, B. G., 2000. Multiple sclerosis. N Engl J Med 343, 938-952.
Novgorodov, A. S., El-Alwani, M., Bielawski, J., Obeid, L. M., Gudz, T. I., 2007. Activation of sphingosine-1-phosphate receptor S1P5 inhibits oligodendrocyte progenitor migration. FASEB J 21, 1503-1514.
Nunes, J., Naymark, M., Sauer, L., Muhammad, A., Keun, H., Sturge, J., Stebbing, J., Waxman, J., Pchejetski, D., 2012. Circulating sphingosine-1-phosphate and erythrocyte sphingosine kinase-1 activity as novel biomarkers for early prostate cancer detection. Br J Cancer 106, 909-915.
Obar, J. J., Lefrancois, L., 2010. Memory CD8+ T cell differentiation. Ann N Y Acad Sci 1183, 251-266.
Ogata, A., Tanaka, T., 2012. Tocilizumab for the treatment of rheumatoid arthritis and other systemic autoimmune diseases: current perspectives and future directions. Int J Rheumatol 2012, 946048.
Oksenberg, J. R., Barcellos, L. F., Cree, B. A., Baranzini, S. E., Bugawan, T. L., Khan, O., Lincoln, R. R., Swerdlin, A., Mignot, E., Lin, L., Goodin, D., Erlich, H. A., Schmidt, S., Thomson, G., Reich, D. E., Pericak-Vance, M. A., Haines, J. L., Hauser, S. L., 2004. Mapping multiple sclerosis susceptibility to the HLA-DR locus in African Americans. Am J Hum Genet 74, 160-167.
Oliet, S. H., Piet, R., Poulain, D. A., 2001. Control of glutamate clearance and synaptic efficacy by glial coverage of neurons. Science 292, 923-926.
Olivera, A., Kohama, T., Edsall, L., Nava, V., Cuvillier, O., Poulton, S., Spiegel, S., 1999. Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. J Cell Biol 147, 545-558.
Olivera, A., Spiegel, S., 2001. Sphingosine kinase: a mediator of vital cellular functions. Prostaglandins 64, 123-134.
Oo, M. L., Chang, S. H., Thangada, S., Wu, M. T., Rezaul, K., Blaho, V., Hwang, S. I., Han, D. K., Hla, T., 2011. Engagement of S1P-degradative mechanisms leads to vascular leak in mice. J Clin Invest 121, 2290-2300.
Oo, M. L., Thangada, S., Wu, M. T., Liu, C. H., Macdonald, T. L., Lynch, K. R., Lin, C. Y., Hla, T., 2007. Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and

proteasomal degradation of the receptor. J Biol Chem 282, 9082-9089.
Osinde, M., Mullershausen, F., Dev, K. K., 2007. Phosphorylated FTY720 stimulates ERK phosphorylation in astrocytes via S1P receptors. Neuropharmacology 52, 1210-1218.
Oyama, Y., Chikahisa, L., Kanemaru, K., Nakata, M., Noguchi, S., Nagano, T., Okazaki, E., Hirata, A., 1998. Cytotoxic actions of FTY720, a novel immunosuppressant, on thymocytes and brain neurons dissociated from the rat. Jpn J Pharmacol 76, 377-385.
Paintlia, M. K., Paintlia, A. S., Singh, A. K., Singh, I., 2011. Synergistic activity of interleukin-17 and tumor necrosis factor-alpha enhances oxidative stress-mediated oligodendrocyte apoptosis. J Neurochem 116, 508-521.
Palmer, A. M., 2010. The role of the blood brain barrier in neurodegenerative disorders and their treatment. J Alzheimers Dis 24, 643-656.
Pan, S., Ryu, S. Y., Sheu, S. S., 2011. Distinctive characteristics and functions of multiple mitochondrial Ca2+ influx mechanisms. Sci China Life Sci 54, 763-769.
Paolinelli, R., Corada, M., Orsenigo, F., Dejana, E., 2011. The molecular basis of the blood brain barrier differentiation and maintenance. Is it still a mystery? Pharmacol Res 63, 165-171.
Pappu, R., Schwab, S. R., Cornelissen, I., Pereira, J. P., Regard, J. B., Xu, Y., Camerer, E., Zheng, Y. W., Huang, Y., Cyster, J. G., Coughlin, S. R., 2007. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science 316, 295-298.
Parekh, A. B., Penner, R., 1997. Store depletion and calcium influx. Physiol Rev 77, 901-930.
Parekh, A. B., Putney, J. W., Jr., 2005. Store-operated calcium channels. Physiol Rev 85, 757-810.
Parish, I. A., Kaech, S. M., 2009. Diversity in CD8(+) T cell differentiation. Curr Opin Immunol 21, 291-297.
Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C., 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 6, 1133-1141.
Parpura, V., Zorec, R., 2010. Gliotransmission: Exocytotic release from astrocytes. Brain Res Rev 63, 83-92.
Parri, H. R., Gould, T. M., Crunelli, V., 2001. Spontaneous astrocytic Ca2+ oscillations in situ drive NMDAR-mediated neuronal excitation. Nat Neurosci 4, 803-812.
Parrill, A. L., Lima, S., Spiegel, S., 2012. Structure of the first sphingosine 1-phosphate receptor. Sci Signal 5, pe23.
Paugh, S. W., Paugh, B. S., Rahmani, M., Kapitonov, D., Almenara, J. A., Kordula, T., Milstien, S., Adams, J. K., Zipkin, R. E., Grant, S., Spiegel, S., 2008. A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. Blood 112, 1382-1391.
Payne, S. G., Milstien, S., Spiegel, S., 2002. Sphingosine-1-phosphate: dual messenger functions. FEBS Lett 531, 54-57.
Pease, J. E., 2011. Targeting chemokine receptors in allergic disease. Biochem J 434, 11-24.
Pebay, A., Toutant, M., Premont, J., Calvo, C. F., Venance, L., Cordier, J., Glowinski, J., Tence, M., 2001. Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. Eur J Neurosci 13, 2067-2076.
Perea, G., Araque, A., 2010. GLIA modulates synaptic transmission. Brain Res Rev 63, 93-102.
Perumal, J. S., Caon, C., Hreha, S., Zabad, R., Tselis, A., Lisak, R., Khan, O., 2008. Oral prednisone taper following intravenous steroids fails to improve disability or recovery from relapses in multiple sclerosis. Eur J Neurol 15, 677-680.
Pham, T. H., Okada, T., Matloubian, M., Lo, C. G., Cyster, J. G., 2008. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. Immunity 28, 122-133.
Pigard, N., Elovaara, I., Kuusisto, H., Paalavuo, R., Dastidar, P., Zimmermann, K., Schwarz, H. P., Reipert, B., 2009. Therapeutic activities of

intravenous immunoglobulins in multiple sclerosis involve modulation of chemokine expression. J Neuroimmunol 209, 114-120.
Pilz, G., Wipfler, P., Ladurner, G., Kraus, J., 2008. Modern multiple sclerosis treatment - what is approved, what is on the horizon. Drug Discov Today 13, 1013-1025.
Pinschewer, D. D., Ochsenbein, A. F., Odermatt, B., Brinkmann, V., Hengartner, H., Zinkernagel, R. M., 2000. FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. J Immunol 164, 5761-5770.
Porter, J. T., McCarthy, K. D., 1996. Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16, 5073-5081.
Proudfoot, A. E., Fritchley, S., Borlat, F., Shaw, J. P., Vilbois, F., Zwahlen, C., Trkola, A., Marchant, D., Clapham, P. R., Wells, T. N., 2001. The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. J Biol Chem 276, 10620-10626.
Pugliatti, M., Rosati, G., Carton, H., Riise, T., Drulovic, J., Vecsei, L., Milanov, I., 2006. The epidemiology of multiple sclerosis in Europe. Eur J Neurol 13, 700-722.
Puneet, P., Yap, C. T., Wong, L., Lam, Y., Koh, D. R., Moochhala, S., Pfeilschifter, J., Huwiler, A., Melendez, A. J., 2010. SphK1 regulates proinflammatory responses associated with endotoxin and polymicrobial sepsis. Science 328, 1290-1294.
Pyne, N. J., Pyne, S., 2010. Sphingosine 1-phosphate and cancer. Nat Rev Cancer 10, 489-503.
Qin, H., Benveniste, E. N., 2012. ELISA methodology to quantify astrocyte production of cytokines/chemokines in vitro. Methods Mol Biol 814, 235-249.
Rachitskaya, A. V., Hansen, A. M., Horai, R., Li, Z., Villasmil, R., Luger, D., Nussenblatt, R. B., Caspi, R. R., 2008. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. J Immunol 180, 5167-5171.
Raine, C. S., 1994. The Dale E. McFarlin Memorial Lecture: the immunology of the multiple sclerosis lesion. Ann Neurol 36 Suppl, S61-72.
Raivich, G., 2005. Like cops on the beat: the active role of resting microglia. Trends Neurosci 28, 571-573.
Rammohan, K. W., 2010. Emerging multiple sclerosis oral therapies. American Academy of Neurology, 47-53.
Ransohoff, R. M., 1999. Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines. J Neuroimmunol 98, 57-68.
Ransom, B. R., Ransom, C. B., 2012. Astrocytes: multitalented stars of the central nervous system. Methods Mol Biol 814, 3-7.
Rao, T. S., Lariosa-Willingham, K. D., Lin, F. F., Yu, N. C., Tham, C. S., Chun, J., Webb, M., 2004. Growth factor pre-treatment differentially regulates phosphoinositide turnover downstream of lysophospholipid receptor and metabotropic glutamate receptors in cultured rat cerebrocortical astrocytes. International Journal of Developmental Neuroscience 22, 131-135.
Rapacciuolo, A., Suvarna, S., Barki-Harrington, L., Luttrell, L. M., Cong, M., Lefkowitz, R. J., Rockman, H. A., 2003. Protein kinase A and G protein-coupled receptor kinase phosphorylation mediates beta-1 adrenergic receptor endocytosis through different pathways. J Biol Chem 278, 35403-35411.
Reboldi, A., Coisne, C., Baumjohann, D., Benvenuto, F., Bottinelli, D., Lira, S., Uccelli, A., Lanzavecchia, A., Engelhardt, B., Sallusto, F., 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat Immunol 10, 514-523.
Rhen, T., Cidlowski, J. A., 2005. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N Engl J Med 353, 1711-1723.
Rieckmann, P., Toyka, K. V., Bassetti, C., Beer, K., Beer, S., Buettner, U., Chofflon, M., Gotschi-Fuchs, M., Hess, K., Kappos, L., Kesselring, J., Goebels, N., Ludin, H. P., Mattle, H., Schluep, M., Vaney, C., Baumhackl, U., Berger, T., Deisenhammer, F., Fazekas, F.,

Freimuller, M., Kollegger, H., Kristoferitsch, W., Lassmann, H., Markut, H., Strasser-Fuchs, S., Vass, K., Altenkirch, H., Bamborschke, S., Baum, K., Benecke, R., Bruck, W., Dommasch, D., Elias, W. G., Gass, A., Gehlen, W., Haas, J., Haferkamp, G., Hanefeld, F., Hartung, H. P., Heesen, C., Heidenreich, F., Heitmann, R., Hemmer, B., Hense, T., Hohlfeld, R., Janzen, R. W., Japp, G., Jung, S., Jugelt, E., Koehler, J., Kolmel, W., Konig, N., Lowitzsch, K., Manegold, U., Melms, A., Mertin, J., Oschmann, P., Petereit, H. F., Pette, M., Pohlau, D., Pohl, D., Poser, S., Sailer, M., Schmidt, S., Schock, G., Schulz, M., Schwarz, S., Seidel, D., Sommer, N., Stangel, M., Stark, E., Steinbrecher, A., Tumani, H., Voltz, R., Weber, F., Weinrich, W., Weissert, R., Wiendl, H., Wietholter, H., Wildemann, U., Zettl, U. K., Zipp, F., Zschenderlein, R., Izquierdo, G., Kirjazovas, A., Packauskas, L., Miller, D., Koncan Vracko, B., Millers, A., Orologas, A., Panellus, M., Sindic, C. J., Bratic, M., Svraka, A., Vella, N. R., Stelmasiak, Z., Selmaj, K., Bartosik-Psujik, H., Mitosek-Szewczyk, K., Belniak, E., Mochecka, A., Bayas, A., Chan, A., Flachenecker, P., Gold, R., Kallmann, B., Leussink, V., Maurer, M., Ruprecht, K., Stoll, G., Weilbach, F. X., 2004. Escalating immunotherapy of multiple sclerosis--new aspects and practical application. J Neurol 251, 1329-1339.

Rommer, P. S., Stuve, O., Goertsches, R., Mix, E., Zettl, U. K., 2008. Monoclonal antibodies in the therapy of multiple sclerosis: an overview. J Neurol 255 Suppl 6, 28-35.

Rosen, H., Gonzalez-Cabrera, P. J., Sanna, M. G., Brown, S., 2009. Sphingosine 1-phosphate receptor signaling. Annu Rev Biochem 78, 743-768.

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M. J., Hauschildt, S., 2011. LPS-induced cytokine production in human monocytes and macrophages. Crit Rev Immunol 31, 379-446.

Rotstein, Z., Hazan, R., Barak, Y., Achiron, A., 2006. Perspectives in multiple sclerosis health care: special focus on the costs of multiple sclerosis. Autoimmun Rev 5, 511-516.

Rouach, N., Pebay, A., Meme, W., Cordier, J., Ezan, P., Etienne, E., Giaume, C., Tence, M., 2006. S1P inhibits gap junctions in astrocytes: involvement of G and Rho GTPase/ROCK. Eur J Neurosci 23, 1453-1464.

Rouvier, E., Luciani, M. F., Mattei, M. G., Denizot, F., Golstein, P., 1993. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. J Immunol 150, 5445-5456.

Rovaris, M., Comi, G., Rocca, M. A., Valsasina, P., Ladkani, D., Pieri, E., Weiss, S., Shifroni, G., Wolinsky, J. S., Filippi, M., 2007. Long-term follow-up of patients treated with glatiramer acetate: a multicentre, multinational extension of the European/Canadian double-blind, placebo-controlled, MRI-monitored trial. Mult Scler 13, 502-508.

Rozemuller, J. M., van Muiswinkel, F. L., 2000. Microglia and neurodegeneration. Eur J Clin Invest 30, 469-470.

Ruggieri, M., Avolio, C., Livrea, P., Trojano, M., 2007. Glatiramer acetate in multiple sclerosis: a review. CNS Drug Rev 13, 178-191.

Sallusto, F., Geginat, J., Lanzavecchia, A., 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 22, 745-763.

Salomon, Y., 1979. Adenylate cyclase assay. Adv Cyclic Nucleotide Res 10, 35-55.

Samoilova, E. B., Horton, J. L., Hilliard, B., Liu, T. S., Chen, Y., 1998. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. J Immunol 161, 6480-6486.

Sanchez, T., Estrada-Hernandez, T., Paik, J. H., Wu, M. T., Venkataraman, K., Brinkmann, V., Claffey, K., Hla, T., 2003. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. J Biol Chem 278, 47281-47290.

Sato, K., Ishikawa, K., Ui, M., Okajima, F., 1999. Sphingosine 1-phosphate induces expression of early growth response-1 and fibroblast growth factor-2 through mechanism involving extracellular signal-regulated kinase in astroglial cells. Brain Res Mol Brain Res 74, 182-189.

Sawada, M., 2009. Neuroprotective and toxic changes in microglia in neurodegenerative disease. Parkinsonism Relat Disord 15 Suppl 1, S39-41.

Schmid, G., Guba, M., Ischenko, I., Papyan, A., Joka, M., Schrepfer, S., Bruns, C. J., Jauch, K. W., Heeschen, C., Graeb, C., 2007. The immunosuppressant FTY720 inhibits tumor angiogenesis via the sphingosine 1-phosphate receptor 1. J Cell Biochem 101, 259-270.

Schroder, M., Richter, C., Juan, M. H., Maltusch, K., Giegold, O., Quintini, G., Pfeilschifter, J. M., Huwiler, A., Radeke, H. H., 2011. The sphingosine kinase 1 and S1P1 axis specifically counteracts LPS-induced IL-12p70 production in immune cells of the spleen. Mol Immunol 48, 1139-1148.

Schurer, S. C., Brown, S. J., Gonzalez-Cabrera, P. J., Schaeffer, M. T., Chapman, J., Jo, E., Chase, P., Spicer, T., Hodder, P., Rosen, H., 2008. Ligand-binding pocket shape differences between sphingosine 1-phosphate (S1P) receptors S1P1 and S1P3 determine efficiency of chemical probe identification by ultrahigh-throughput screening. ACS Chem Biol 3, 486-498.

Schwid, S. R., Panitch, H. S., 2007. Full results of the Evidence of Interferon Dose-Response-European North American Comparative Efficacy (EVIDENCE) study: a multicenter, randomized, assessor-blinded comparison of low-dose weekly versus high-dose, high-frequency interferon beta-1a for relapsing multiple sclerosis. Clin Ther 29, 2031-2048.

Sharief, M. K., Hentges, R., 1991. Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. N Engl J Med 325, 467-472.

Shenoy, S. K., McDonald, P. H., Kohout, T. A., Lefkowitz, R. J., 2001. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. Science 294, 1307-1313.

Sheridan, G. K., Dev, K. K., 2012. S1P1 receptor subtype inhibits demyelination and regulates chemokine release in cerebellar slice cultures. Glia 60, 382-392.

Shi, N., Kawano, Y., Matsuoka, T., Mei, F., Ishizu, T., Ohyagi, Y., Kira, J., 2009. Increase of CD4+TNF{alpha}+IL-2-T cells in cerebrospinal fluid of multiple sclerosis patients. Mult Scler 15, 120-123.

Shiina, T., Nagao, T., Kurose, H., 2001. Low affinity of beta1-adrenergic receptor for beta-arrestins explains the resistance to agonist-induced internalization. Life Sci 68, 2251-2257.

Sidoryk-Wegrzynowicz, M., Wegrzynowicz, M., Lee, E., Bowman, A. B., Aschner, M., 2011. Role of astrocytes in brain function and disease. Toxicol Pathol 39, 115-123.

Singaravelu, K., Lohr, C., Deitmer, J. W., 2006. Regulation of store-operated calcium entry by calcium-independent phospholipase A2 in rat cerebellar astrocytes. J Neurosci 26, 9579-9592.

Singleton, P. A., Dudek, S. M., Ma, S. F., Garcia, J. G., 2006. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family. J Biol Chem 281, 34381-34393.

Siow, D. L., Anderson, C. D., Berdyshev, E. V., Skobeleva, A., Natarajan, V., Pitson, S. M., Wattenberg, B. W., 2011. Sphingosine kinase localization in the control of sphingolipid metabolism. Adv Enzyme Regul 51, 229-244.

Slimani, H., Charnaux, N., Mbemba, E., Saffar, L., Vassy, R., Vita, C., Gattegno, L., 2003. Interaction of RANTES with syndecan-1 and syndecan-4 expressed by human primary macrophages. Biochim Biophys Acta 1617, 80-88.

Smith, K. J., Lassmann, H., 2002. The role of nitric oxide in multiple sclerosis. Lancet Neurol 1, 232-241.

Sofroniew, M. V., Vinters, H. V., 2010. Astrocytes: biology and pathology. Acta Neuropathol 119, 7-35.

Sorensen, S. D., Nicole, O., Peavy, R. D., Montoya, L. M., Lee, C. J., Murphy, T. J., Traynelis, S. F., Hepler, J. R., 2003. Common signaling pathways link activation of murine PAR-1, LPA, and S1P receptors to proliferation of astrocytes. Mol Pharmacol 64, 1199-1209.

Sospedra, M., Martin, R., 2005. Immunology of multiple sclerosis. Annu Rev Immunol 23, 683-747.

Spiegel, S., Milstien, S., 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol 4, 397-407.
Spiegel, S., Milstien, S., 2011. The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol 11, 403-415.
Struyf, S., Menten, P., Lenaerts, J. P., Put, W., D'Haese, A., De Clercq, E., Schols, D., Proost, P., Van Damme, J., 2001. Diverging binding capacities of natural LD78beta isoforms of macrophage inflammatory protein-1alpha to the CC chemokine receptors 1, 3 and 5 affect their anti-HIV-1 activity and chemotactic potencies for neutrophils and eosinophils. Eur J Immunol 31, 2170-2178.
Sutton, C., Brereton, C., Keogh, B., Mills, K. H., Lavelle, E. C., 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med 203, 1685-1691.
Szczucinski, A., Losy, J., 2011. CCL5, CXCL10 and CXCL11 chemokines in patients with active and stable relapsing-remitting multiple sclerosis. Neuroimmunomodulation 18, 67-72.
Takata, N., Hirase, H., 2008. Cortical layer 1 and layer 2/3 astrocytes exhibit distinct calcium dynamics in vivo. PLoS One 3, e2525.
Tas, P. W., Koschel, K., 1998. Sphingosine-1-phosphate induces a Ca2+ signal in primary rat astrocytes and a Ca2+ signal and shape changes in C6 rat glioma cells. J Neurosci Res 52, 427-434.
Tham, C. S., Lin, F. F., Rao, T. S., Yu, N., Webb, M., 2003. Microglial activation state and lysophospholipid acid receptor expression. Int J Dev Neurosci 21, 431-443.
Thrower, B. W., 2007. Clinically isolated syndromes: predicting and delaying multiple sclerosis. Neurology 68, S12-15.
Toman, R. E., Payne, S. G., Watterson, K. R., Maceyka, M., Lee, N. H., Milstien, S., Bigbee, J. W., Spiegel, S., 2004. Differential transactivation of sphingosine-1-phosphate receptors modulates NGF-induced neurite extension. J Cell Biol 166, 381-392.
Tourbah, A., Lyon-Caen, O., 2007. Interferons in multiple sclerosis: ten years' experience. Biochimie 89, 899-902.
Trajkovic, V., Stosic-Grujicic, S., Samardzic, T., Markovic, M., Miljkovic, D., Ramic, Z., Mostarica Stojkovic, M., 2001. Interleukin-17 stimulates inducible nitric oxide synthase activation in rodent astrocytes. J Neuroimmunol 119, 183-191.
Traugott, U., Reinherz, E. L., Raine, C. S., 1983. Multiple sclerosis: distribution of T cell subsets within active chronic lesions. Science 219, 308-310.
Trebst, C., Sorensen, T. L., Kivisakk, P., Cathcart, M. K., Hesselgesser, J., Horuk, R., Sellebjerg, F., Lassmann, H., Ransohoff, R. M., 2001. CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. Am J Pathol 159, 1701-1710.
Trejo, J., Coughlin, S. R., 1999. The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. J Biol Chem 274, 2216-2224.
Tzartos, J. S., Friese, M. A., Craner, M. J., Palace, J., Newcombe, J., Esiri, M. M., Fugger, L., 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol 172, 146-155.
Uhlen, P., Fritz, N., 2010. Biochemistry of calcium oscillations. Biochem Biophys Res Commun 396, 28-32.
Vallabhapurapu, S., Karin, M., 2009. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 27, 693-733.
Van Doorn, R., Van Horssen, J., Verzijl, D., Witte, M., Ronken, E., Van Het Hof, B., Lakeman, K., Dijkstra, C. D., Van Der Valk, P., Reijerkerk, A., Alewijnse, A. E., Peters, S. L., De Vries, H. E., 2010. Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. Glia 58, 1465-1476.
van Loo, G., De Lorenzi, R., Schmidt, H., Huth, M., Mildner, A., Schmidt-Supprian, M., Lassmann, H., Prinz, M. R., Pasparakis, M., 2006. Inhibition of transcription factor NF-kappaB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. Nat Immunol 7, 954-961.
van Neerven, S., Regen, T., Wolf, D., Nemes, A., Johann, S., Beyer, C., Hanisch, U. K., Mey, J., 2010. Inflammatory chemokine release of

astrocytes in vitro is reduced by all-trans retinoic acid. J Neurochem 114, 1511-1526.
Van Wagoner, N. J., Oh, J. W., Repovic, P., Benveniste, E. N., 1999. Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor. J Neurosci 19, 5236-5244.
Verkhratsky, A., Rodriguez, J. J., Parpura, V., 2012. Calcium signalling in astroglia. Mol Cell Endocrinol 353, 45-56.
Wang, D. D., Bordey, A., 2008. The astrocyte odyssey. Prog Neurobiol 86, 342-367.
Wang, L., Dudek, S. M., 2009. Regulation of vascular permeability by sphingosine 1-phosphate. Microvasc Res 77, 39-45.
Wang, Y., Zhou, Y., Szabo, K., Haft, C. R., Trejo, J., 2002. Down-regulation of protease-activated receptor-1 is regulated by sorting nexin 1. Mol Biol Cell 13, 1965-1976.
Warnock, R. A., Askari, S., Butcher, E. C., von Andrian, U. H., 1998. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. J Exp Med 187, 205-216.
Watford, W. T., Moriguchi, M., Morinobu, A., O'Shea, J. J., 2003. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 14, 361-368.
Watterson, K. R., Johnston, E., Chalmers, C., Pronin, A., Cook, S. J., Benovic, J. L., Palmer, T. M., 2002. Dual regulation of EDG1/S1P(1) receptor phosphorylation and internalization by protein kinase C and G-protein-coupled receptor kinase 2. J Biol Chem 277, 5767-5777.
Watts, C., 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nat Immunol 5, 685-692.
Waubant, E., 2012. Early recognition and diagnosis of multiple sclerosis. J Clin Psychiatry 73, e14.
Webb, M., Tham, C. S., Lin, F. F., Lariosa-Willingham, K., Yu, N., Hale, J., Mandala, S., Chun, J., Rao, T. S., 2004. Sphingosine 1-phosphate receptor agonists attenuate relapsing-remitting experimental autoimmune encephalitis in SJL mice. J Neuroimmunol 153, 108-121.
Weber, M. S., Hohlfeld, R., Zamvil, S. S., 2007. Mechanism of action of glatiramer acetate in treatment of multiple sclerosis. Neurotherapeutics 4, 647-653.
Weiner, H. L., Cohen, J. A., 2002. Treatment of multiple sclerosis with cyclophosphamide: critical review of clinical and immunologic effects. Mult Scler 8, 142-154.
Weissert, R., 2011. Progressive multifocal leukoencephalopathy. J Neuroimmunol 231, 73-77.
Wekerle, H., 2008. Lessons from multiple sclerosis: models, concepts, observations. Ann Rheum Dis 67 Suppl 3, iii56-60.
Westby, M., van der Ryst, E., 2010. CCR5 antagonists: host-targeted antiviral agents for the treatment of HIV infection, 4 years on. Antivir Chem Chemother 20, 179-192.
Willer, C. J., Dyment, D. A., Risch, N. J., Sadovnick, A. D., Ebers, G. C., 2003. Twin concordance and sibling recurrence rates in multiple sclerosis. Proc Natl Acad Sci U S A 100, 12877-12882.
Witowski, J., Ksiazek, K., Jorres, A., 2004. Interleukin-17: a mediator of inflammatory responses. Cell Mol Life Sci 61, 567-579.
Wu, Y. P., Mizugishi, K., Bektas, M., Sandhoff, R., Proia, R. L., 2008. Sphingosine kinase 1/S1P receptor signaling axis controls glial proliferation in mice with Sandhoff disease. Human Molecular Genetics 17, 2257-2264.
Xiang, Y., Hirth, B., Kane, J. L., Jr., Liao, J., Noson, K. D., Yee, C., Asmussen, G., Fitzgerald, M., Klaus, C., Booker, M., 2010. Discovery of novel sphingosine kinase-1 inhibitors. Part 2. Bioorg Med Chem Lett 20, 4550-4554.
Xie, W., Strong, J. A., Kays, J., Nicol, G. D., Zhang, J. M., 2012. Knockdown of the sphingosine-1-phosphate receptor S1PR1reduces pain behaviors induced by local inflammation of the rat sensory ganglion. Neurosci Lett 515, 61-65.
Xu, M., Waters, C. L., Hu, C., Wysolmerski, R. B., Vincent, P. A., Minnear, F. L., 2007. Sphingosine 1-phosphate rapidly increases endothelial barrier function independently of VE-cadherin but requires cell spreading and Rho kinase. Am J Physiol Cell Physiol 293, C1309-1318.

Yamagata, K., Tagami, M., Torii, Y., Takenaga, F., Tsumagari, S., Itoh, S., Yamori, Y., Nara, Y., 2003. Sphingosine 1-phosphate induces the production of glial cell line-derived neurotrophic factor and cellular proliferation in astrocytes. Glia 41, 199-206.

Yan, H. Q., Banos, M. A., Herregodts, P., Hooghe, R., Hooghe-Peters, E. L., 1992. Expression of interleukin (IL)-1 beta, IL-6 and their respective receptors in the normal rat brain and after injury. Eur J Immunol 22, 2963-2971.

Yang, J. S., Xu, L. Y., Xiao, B. G., Hedlund, G., Link, H., 2004. Laquinimod (ABR-215062) suppresses the development of experimental autoimmune encephalomyelitis, modulates the Th1/Th2 balance and induces the Th3 cytokine TGF-beta in Lewis rats. J Neuroimmunol 156, 3-9.

Yao, Z., Fanslow, W. C., Seldin, M. F., Rousseau, A. M., Painter, S. L., Comeau, M. R., Cohen, J. I., Spriggs, M. K., 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 3, 811-821.

Yeh, E. A., Weinstock-Guttman, B., 2011. Fingolimod: an oral disease-modifying therapy for relapsing multiple sclerosis. Adv Ther 28, 270-278.

Yong, V. W., Power, C., Forsyth, P., Edwards, D. R., 2001. Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2, 502-511.

Yoon, C. M., Hong, B. S., Moon, H. G., Lim, S., Suh, P. G., Kim, Y. K., Chae, C. B., Gho, Y. S., 2008. Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. Blood 112, 1129-1138.

Young, K. W., Bootman, M. D., Channing, D. R., Lipp, P., Maycox, P. R., Meakin, J., Challiss, R. A., Nahorski, S. R., 2000. Lysophosphatidic acid-induced Ca2+ mobilization requires intracellular sphingosine 1-phosphate production. Potential involvement of endogenous EDG-4 receptors. J Biol Chem 275, 38532-38539.

Yousry, T. A., Major, E. O., Ryschkewitsch, C., Fahle, G., Fischer, S., Hou, J., Curfman, B., Miszkiel, K., Mueller-Lenke, N., Sanchez, E., Barkhof, F., Radue, E. W., Jager, H. R., Clifford, D. B., 2006. Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. N Engl J Med 354, 924-933.

Yu, N., Lariosa-Willingham, K. D., Lin, F. F., Webb, M., Rao, T. S., 2004. Characterization of lysophosphatidic acid and sphingosine-1-phosphate-mediated signal transduction in rat cortical oligodendrocytes. Glia 45, 17-27.

Zeinstra, E., Wilczak, N., De Keyser, J., 2003. Reactive astrocytes in chronic active lesions of multiple sclerosis express co-stimulatory molecules B7-1 and B7-2. J Neuroimmunol 135, 166-171.

Zepp, J., Wu, L., Li, X., 2011. IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease. Trends Immunol 32, 232-239.

Zhang, Y., Zhai, Q., Luo, Y., Dorf, M. E., 2002. RANTES-mediated chemokine transcription in astrocytes involves activation and translocation of p90 ribosomal S6 protein kinase (RSK). J Biol Chem 277, 19042-19048.

Zhang, Z., Fauser, U., Schluesener, H. J., 2008. Early attenuation of lesional interleukin-16 up-regulation by dexamethasone and FTY720 in experimental traumatic brain injury. Neuropathol Appl Neurobiol 34, 330-339.

Zhao, Q., 2010. Dual targeting of CCR2 and CCR5: therapeutic potential for immunologic and cardiovascular diseases. J Leukoc Biol 88, 41-55.

Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., Abbas, A. R., Modrusan, Z., Ghilardi, N., de Sauvage, F. J., Ouyang, W., 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med 14, 282-289.

Zorec, R., Araque, A., Carmignoto, G., Haydon, P. G., Verkhratsky, A., Parpura, V., 2012. Astroglial excitability and gliotransmission: an appraisal of Ca2+ as a signalling route. ASN Neuro 4.