Dual agonist-antagonist functions of FTY720 influence neuroinflammation-relevant responses in human astrocytes

Submitted by Celina Wu, Neuroimmunology Unit, Integrated Program in Neuroscience,

McGill University, Montreal

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Abstract

Astrocytes are the most abundant glia in the central nervous system (CNS), classically identified by their high expression of the intermediate filament, glial fibrillary acidic protein (GFAP). Astrocytes participate in a number of biochemical events important for CNS functions and play a dynamic role in regulating CNS injury/repair processes. In chronic inflammatory conditions such as multiple sclerosis (MS), astrocytes undergo pathophysiological changes that lead to a feature termed astrogliosis (Liberto, Albrecht et al. 2004; Sidoryk-Wegrzynowicz, Wegrzynowicz et al. 2011). Astrogliosis is common to MS lesions, and a novel therapeutic agent for MS, FTY720 (fingolimod, Gilenya™) demonstrates neuroprotective potential by inhibiting astrogliosis development (Choi, Gardell et al. 2011). FTY720 is an oral therapy recently approved for the treatment of MS, and is shown to readily access the CNS. There, it binds directly to sphingosine-1-phosphate receptors (S1PR) on astrocytes and the dynamics of S1PR signaling is shown to modulate astrocytic cellular responses that closely relate to MS pathology. This thesis examines the signaling and functional effects of FTY720 on primary human astrocytes. We used astrocytes derived from the human fetal CNS to explore neuroinflammation-relevant responses mediated by chronic (repeated daily) FTY720 administrations. FTY720 is known to initially acts as an agonist, activating S1PRs but also functions as an antagonist by promoting S1PR internalization and degradation; we examined whether these effects occurred in tandem. We report that receptors internalized by FTY720 can persist and continue to signal for an extended time period (hours). A single addition of FTY720 desensitizes the extracellular receptor-regulated phosphorylation (pERK)

signaling response for >24 hours. Such refractory period for pERK signal transduction was maintained in astrocytes treated repeatedly (daily) with FTY720, otherwise the return of pERK activation was achieved by 72 hours following initial treatment. Moreover, receptor desensitization patterns correlated with the loss of proliferative responses induced by the natural ligand sphingosine-1-phosphate (S1P). We show that even under the condition of receptor desensitization (repeated daily administrations) FTY720 attenuated the capacity of the pro-inflammatory cytokine IL-1β, to activate calcium sensitive pathways. Repeated FTY720 treatments did not inhibit serum-induced pERK responses or the secretions of IL-6 and IP-10 in response to IL-1β activation. Our results indicate that daily FTY720 exposures can be a relevant regulator of neuro-inflammation by acting as a functional antagonist for external stimuli (natural ligand S1P) while sustaining internalized receptor-dependent agonist functions (inhibit IL-1β induced calcium mobilization).

Résumé

Les astrocytes sont les cellules gliales les plus abondantes du système nerveux central (SNC). Leur grande expression en filaments intermédiaires, la protéine acide fibrillaire gliale (GFAP), est une caractéristique permettant leur identification. Les astrocytes sont d'importants contributeurs aux événements biochimiques du SNC et jouent un rôle clé dans le processus de régulation des dommages et de la guérison du SNC. Sous des conditions d'inflammation chronique, tel la Sclérose en Plaques (SP), les astrocytes subissent des changements pathophysiologiques causant l'astrogliose (Liberto, Albrecht et al. 2004; Sidoryk-Wegrzynowicz, Wegrzynowicz et al. 2011). Ce mécanisme de cicatrisation est commun dans la SP et un nouvel agent thérapeutique, FTY720 (fingolimod, Gilenya™) démontre des effets protecteurs du SNC en prévenant l'évolution de l'astrogliose. (Choi, Gardell et al. 2011). FTY720 est un agent thérapeutique récemment approuvé pour traiter la SP. Il est administré oralement et a la capacité d'accéder au SNC. Une fois en place dans ce système, cet agent entre en contact direct avec le récepteur sphingosine-1-phosphate (S1PR) sur les astrocytes. Les réponses des astrocytes en réaction aux signaux générés par ce récepteur sont reliées à la pathologie de la SP. Cette thèse examine les signaux engendrés par FTY720 ainsi que ses fonctions sur les astrocytes humains primaires. Nous avons utilisé des astrocytes isolés à partir de SNC humains fœtaux pour examiner les réponses neuro-inflammatoires générées par l'administration quotidienne de FTY720. FTY720 agit initialement comme un agoniste en activant le récepteur S1P, mais il agit également comme un antagoniste en causant l'internalisation et la dégradation de ce récepteur. Nous avons examiné ces deux

phénomènes de façon à savoir s'ils agissent en concert. Nous affirmons qu'un récepteur internalisé par FTY720 continue de générer des signaux pour une période de temps prolongée (heures). Une addition simple de FTY720 désensibilise l'astrocyte, pour une période de >24h, au signal de phosphorylation de ERK (pERK) qui est généré par le récepteur extracellulaire. Cette période réfractaire du signal de transduction de pERK fût maintenue dans les astrocytes traités quotidiennement avec FTY720, sinon le signal pERK reparaît 72 heures après le traitement initial. De plus, la désensibilisation du récepteur fût reliée à l'absence de réponse proliférative induite par le ligand naturel sphingosine-1-phosphate (S1P). Nous avons aussi démontré que le traitement quotidien des astrocytes avec FTY720 atténue la capacité de IL-1β à activer les voies moléculaires sensibles au calcium. Le traitement quotidien avec FTY720 n'inhibe pas les signaux de pERK lorsque les astrocytes sont stimulés à l'aide de sérum, ni la sécrétion de IL-6 ou de IP-10 lorsqu'ils sont stimulés avec IL-1β. Nos résultats suggèrent que l'exposition quotidienne à FTY720 agit comme un antagoniste aux stimuli extérieur (tel le ligand naturel S1P) ainsi qu'un agoniste lorsque le récepteur est internalisé (inhibe la mobilisation du calcium lorsqu'exposé à IL-1β).

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Contributions of Authors

The candidate developed the experimental designs independently and conducted the studies with assistance from the laboratory technicians, Manon Blain and Ellie McCrea. Dr. Jack Antel, Bryce Durafort, David Henault and Dr. Craig S. Moore provided editorial insights for the manuscript preparation.

List of Abbreviations

BBB	blood brain barrier
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CT	cycle thresholds
EAE	experimental autoimmune encephalomyelitis
ER	endoplasmic reticulum
FCS	fetal calf serum
FTY720	2-amino-2-[2-(4-octyl-phenyl)ethyl]-1, 3-propanediol hydrochloride
GFAP	glial fibrillary acidic protein
HFA	human fetal astrocytes
IFNϒ	interferon gamma
iNOS	inducible nitric oxide synthase
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IP3	inositol trisphosphate
IP-10	interferon gamma-induced protein 10
MCP-1	monocyte chemotactic protein-1
MS	multiple sclerosis
pERK	phospho-extracellular signal-regulated kinases
PKC	protein kinase C
PLC	phospholipase C
RT-PCR	real-time polymerase chain reaction
S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
TNFα	tumor necrosis factor alpha

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Introduction

5.1. Background of multiple sclerosis

Multiple sclerosis (MS) is an inflammatory disease of the brain and spinal cord, and the main etiology remains unknown. A number of factors have been implicated for MS, as it is thought to be an autoimmune disease that develops in response to a combination of non-mutually exclusive factors including genetics, environmental factors, diet and infections (reviewed in Corthals 2011). Many of these observations derive from the histopathology of MS encompassing a number of classical hallmarks; distinct features of MS include demyelination, axonal injury, substantial glial scarring and the infiltration of peripheral immune cells into the central nervous system (CNS) (Sorensen, Trebst et al. 2002; Chun and Hartung 2010; Dutta and Trapp 2011).

The most prominent feature of MS is demyelination. Demyelination occurs with thinning or damage to the myelin surrounding neuronal axons, and the cells involved in myelination are oligodendrocytes. Oligodendrocytes are the main cells targeted for autodestruction while other cell types experience concomitant bystander injury (Dutta and Trapp 2011). First, demyelination decreases neural electrical conductivity and exposes neuronal axons to environmental products (Dutta and Trapp 2011). As such, axons become predisposed to immune-mediated transection thereby interrupting signal transduction (Dutta and Trapp 2011). Another cell type considerably affected in MS is astrocytes (Van Doorn, Van Horssen et al. 2010; Choi, Gardell et al. 2011). Astrocytes participate in a number of biochemical events important for CNS functions (Pekny and

Pekna 2004). Among them, astrocytes contribute to the structural and functional integrity of the blood-brain-barrier (BBB), and regulate blood flow and energy metabolism within the CNS (Prat, Biernacki et al. 2001). Other homeostatic processes modulated by astrocytes include ion and water exchange, and the uptake of glutamate during neurotransmission (Nag 2011). Disturbances in astrocyte function are shown to contribute to neurodegeneration, and an increasing number of studies demonstrate that the health of astrocytes may serve as a biomarker for MS reactivity (Nair, Frederick et al. 2008; Choi, Gardell et al. 2011). In the context of neuroinflammation, astrocytes play a dynamic role in regulating CNS injury/repair processes; astrocytes can secrete neurotrophic factors permissive to neuro-regeneration or hinder CNS repair by transforming into reactive astrocytes leading to astrogliosis (Liberto, Albrecht et al. 2004; Sidoryk-Wegrzynowicz, Wegrzynowicz et al. 2011).

5.2. Reactive astrocytes and therapeutic targets

Astrogliosis is described as activated astrocytes accompanied by excessive proliferation and morphological changes (hypertrophy, process extension and GFAP over-expression) (Pekny and Pekna 2004). Activated astrocytes modulate the activity of surrounding cells, and can promote CNS inflammation by regulating its surface marker expressions, secreting soluble factors and influencing the integrity of the BBB (Nair, Frederick et al. 2008). Particularly, astrocytes can secrete chemokines and upregulate adhesion molecules and consequently recruit peripheral lymphocytes to extravasate into the CNS (Biernacki, Prat et al. 2001; Ramgolam, Sha et al. 2009). Blocking lymphocyte

extravasation into the CNS by targeting adhesion markers (metalloproteinase expressions) is one of the effects for Rebif® efficacy, an interferon beta (IFN)-1a therapy currently in clinical use for MS (Markowitz 2007; Ramgolam, Sha et al. 2009).

Another factor that modulates MS activity is the local cytokine/chemokine profiles. Proinflammatory factors are major features of MS pathology, and reactive astrocytes release cytokines and chemokines that propagate activating signals in a paracrine and autocrine manner (reviewed in Nair et al., 2008). Among them, interleukin (IL)-6 is a cytokine prominently secreted by activated astrocytes (Spooren, Kooijman et al. 2010). IL-6 has pleiotrophic effects on the immune and CNS, and is shown to promote pathological proliferation in astrocytes (Selmaj, Farooq et al. 1990). Furthermore, IL-6 plays an important role in experimental autoimmune encephalomyelitis (EAE) induction, an animal model of MS (Eugster, Frei et al. 1998; Dong and Benveniste 2001). IL-6-deficient mice are reported to be resistant to EAE, and exogenously introducing IL-6 reverted EAE susceptibility in the transgenic mice (Eugster, Frei et al. 1998; Dong and Benveniste 2001). Other soluble products implicated for MS include monocyte chemotactic protein-1 (MCP-1) and IFN-gamma-inducible 10-kDa protein (IP-10). Astrocytes are significant CNS producers of these chemokines, and elevated levels may exacerbate MS by recruiting circulating macrophages and autoreactive T cells into the CNS (Ransohoff, Hamilton et al. 1993; Glabinski, Tani et al. 1997). Moreover, numerous cytokines activate astrocytes, particularly those that pertain to the Th1[1] family (Choi, Gardell et al. 2011). For instance, IL-1β receptor activation on astrocytes is known to regulate diverse biological functions that are relevant for inflammation (John, Lee et al.

[1] Th[1] (T helper 1) family: mediators that generate a proinflammatory response to microbicidal antigens. Cytokines classified as belonging to the Th[1] family include IFNϒ, IL-1β and TNFα.

2005). IL-1β receptor activations signal through the NF-kB pathway, and acute stimulation with IL-1β triggers the release of intracellular calcium stores in astrocytes (Holliday and Gruol 1993; Beskina, Miller et al. 2007). As calcium signaling functions as the primary mode of glioexcitability, disrupting calcium homeostasis in astrocytes (including overstimulation) exert a number of pathological responses, including astrocyte activation and apoptosis (Rzigalinski, Liang et al. 1997; Fukuda, Deshpande et al. 1998).

5.3. Sphingolipids and the MS therapy FTY720

More recently, a bioactive membrane phospholipid known as sphingolipid was shown to have a high relevance in MS pathogenesis, namely through the identification of the therapeutic agent, FTY720 (Brinkmann, Davis et al. 2002). FTY720 (fingolimod, Gilenya™) is a structural analog of the endogenous lipid metabolite, sphinosine-1-phosphate (S1P), and was isolated from the *Isaria sinclairii* fungal metabolite, myriocin (Suzuki, Enosawa et al. 1996). FTY720 recently completed its Phase III clinical trials as a treatment for the relapsing-remitting form of MS and is shown to be efficacious in decreasing clinical relapses (Brinkmann, Davis et al. 2002; Chun and Brinkmann 2011). MRI studies show patients on FTY720 experience a reduction in the number and size of lesions, along with a decrease in the rate of brain atrophy (Devonshire, Havrdova et al. 2012).

Biochemically, FTY720 is a 2-amino-2[2-(4-octylphenyl)ethyl] propane-1,3-diol and is phosphorylated into its active form by endogenous sphinosine kinases (Albert, Hinterding et al. 2005; Anada, Igarashi et al. 2007; Foster, Howard et al. 2007). Given its

lipid-solubility, the orally administered FTY720 can penetrate through tissues membranes (including the brain), and become activated by local sphingosine kinases (Olivera, Kohama et al. 1999; Anelli, Bassi et al. 2005; Fischer, Alliod et al. 2011).

Sphingosine kinase and its substrate S1P incite a number of biological processes (including proliferation, migration and survival) in numerous cell types (Bassi, Anelli et al. 2006; Mullershausen, Craveiro et al. 2007; Kimura, Ohmori et al. 2008; Maceyka, Harikumar et al. 2012). S1P is produced primarily by platelets however, local release of S1P by tissues is observed at sites of injury and infection (Bassi, Anelli et al. 2006; Maceyka, Harikumar et al. 2012). In the immune system, S1P functions as a chemotactic factor and S1P-S1P receptor (S1PR) interactions play a pivotal role for lymphocyte trafficking (Brinkmann 2009; Chi 2011). Lymphocyte egression from lymphoid organs and into circulation requires S1PR activation, and is dependent on the S1P gradient between blood and interstitial fluids (Brinkmann, Davis et al. 2002).

FTY720's potent immunomodulatory effects are mediated by functionally antagonizing S1PR activation in lymphocyte subsets (Graler and Goetzl 2004). Blocking S1PR signaling renders lymphocytes sequestered in lymphoid organs and patients treated with 0.5mg FTY720 daily demonstrate significant lymphopenia (Brinkmann, Davis et al. 2002; Graler and Goetzl 2004; Devonshire, Havrdova et al. 2012). Similar to the aforementioned MS therapy Rebif®, the efficacy of FTY720 for treating the disease is primarily by decreasing lymphocyte infiltrating the CNS (Brinkmann, Davis et al. 2002; Dev, Mullershausen et al. 2008).

5.4. Sphingosine-1-phosphate signaling in astrocytes

In addition to its systemic immune-directed effects however, FTY720 readily accesses the CNS and directly binds neuronal cells (Foster, Howard et al. 2007; Dev, Mullershausen et al. 2008). CNS cells ubiquitously express S1P receptors, and the human fetal astrocytes express high levels of S1P1 and S1P3 receptor transcripts under basal conditions (Dev, Mullershausen et al. 2008; Miron, Schubart et al. 2008; Van Doorn, Van Horssen et al. 2010). Modulations of S1PR were shown in response to the presence of proinflammatory cytokines (such as TNFα or IL-1β) (Van Doorn, Van Horssen et al. 2010), although one is urged to take precaution in correlating transcript expression with protein levels.

Choi and colleagues (2011) advocated for the efficacy of FTY720 extending beyond immunological mechanisms; they provided evidence that FTY720 directly modulated the pathogenesis of EAE via S1PR on astrocytes (Choi, Gardell et al. 2011). S1P and FTY720 are cognate ligands for S1PR, however unlike S1P, FTY720 binds four of the five S1PR (S1P1, 3-5) subtypes and with greatest affinity for S1P1R (Brinkmann, Davis et al. 2002; Dev, Mullershausen et al. 2008). Shortly following initial S1PR activation, a bifurcation in signal transduction is observed for FTY720 compared to the natural ligand (Mullershausen, Zecri et al. 2009). This divergence in receptor-activation pathway is hypothesized to be a major factor for their contrasting influences on MS activity, with FTY720 ameliorating the disease and S1P promoting MS pathogenesis (further discussed in 5.7) (Mullershausen, Zecri et al. 2009).

5.5. S1P receptors and signaling dynamics

The 5 subtypes of S1PR (S1P1-5) are G protein coupled receptors (GPCR), each linked to different heterotrimeric guanine nucleotide binding proteins (zu Heringdorf, Vincent et al. 2003; Dev, Mullershausen et al. 2008). Current literature supports the roles of phospholipase C (PLC), extracellular signal regulated kinase (ERK) and cyclic adenosine monophosphate (cAMP) in propagating S1PR activated signals to functional target-sites (zu Heringdorf, Vincent et al. 2003; Giussani, Ferraretto et al. 2007; Osinde, Mullershausen et al. 2007). The pattern of S1PR subtype activation may generate differential signaling cascades responses; in human astrocytes, focus has been given primarily to S1P1 and 3 receptor activation since these cells express low (if any) levels of the remaining receptor subtypes (S1P2, 4, 5R) (Miron, Schubart et al. 2008) (Verzijl, Peters et al. 2010).

The activation of S1P3R results in a manner indicative of multiple G protein coupling to this receptor subtype (Harris, Creason et al. 2012). Most notable is the G_q-activation of an intracellular signaling cascade leading to a rapid rise in calcium (zu Heringdorf, Vincent et al. 2003; Harris, Creason et al. 2012). This calcium raise is blocked by thapsigargin, an endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, suggesting that the calcium efflux observed with receptor activation are from intracellular stores (Grynkiewicz, Poenie et al. 1985; zu Heringdorf, Vincent et al. 2003; Harris, Creason et al. 2012).

As mentioned above, activation of calcium-sensitive pathways in astrocytes has important implications (Rapizzi, Donati et al. 2007; Yoon, Hong et al. 2008; Lee, Choi et

al. 2010). Dal Pra et al. (2005) showed that activation of astrocytes by proinflammatory cytokines such as IL-1β, IFNg, and TNFα induced iNOS expression in a calcium-dependent manner. The authors showed that iNOS induction could be blocked by reducing calcium concentrations (Dal Pra, Chiarini et al. 2005). Furthermore, Holliday and Gruol (1993) provided additional evidence for the effects of IL-1β impacting calcium, as they showed that disruptions in calcium balance (by glutamate activation) were most pronounced when astrocytes were pretreated with IL-1β and forskolin, an activator of adenylyl cyclase.

Calcium mobilization is commonly used as a proxy for S1PR activation, and the absence of calcium elevations to S1P is often attributed as the lack of surface S1PR (Mullershausen, Zecri et al. 2009). zu Heringdorf et al. (2003) however, showed an opposing phenomenon mediated by differential S1PR activation. Their transfection studies showed that S1P elicited calcium mobilization in rat hepatoma cells expressing S1P3R whereas calcium elevations were not detected in cells expressing S1P1R (zu Heringdorf, Vincent et al. 2003). Furthermore, they saw that S1P pretreatment rendered S1P1R-expressing cells less responsive to ATP-evoked calcium efflux (zu Heringdorf, Vincent et al. 2003). They suggested that S1P1R activates PKCα and PKCβI, the negative-regulators of PLC, thereby inhibiting calcium efflux from IP3-dependent ER stores (zu Heringdorf, Vincent et al. 2003). Moreover, they showed that direct activations of these PKCs by phorbol 12-myristate 13-acetate mimicked the inhibitory effect observed with S1P1-G_i activation (zu Heringdorf, Vincent et al. 2003).

The complexities of S1P3R activation are evident, as it demonstrates features of multiple G protein-coupling. S1P1R, on the other hand, is exclusively coupled with $G_{i,}$

and G_i receptor activation results in classical responses including the inhibition of adenylyl cyclase, ERK phosphorylation, and receptor internalization (Pebay, Toutant et al. 2001; zu Heringdorf, Vincent et al. 2003; Mullershausen, Zecri et al. 2009). Shortly following ligand-engagement, G_i-coupled receptors are phosphorylated by G-receptor kinase 2 and a number of complexes are recruited to the plasma membrane for endocytosis initiation (May, Leach et al. 2007). Since S1P3Rs are also coupled to G_i, they too are internalized via G_i-mediated endocytosis, and the duration of receptor desensitization is regulated by processes such as intracellular trafficking or receptor recycling (Verzijl, Peters et al. 2010; Hla and Brinkmann 2011). Acute exposures of the physiological ligand to G_i-coupled S1PR induce receptor recycling from plasma membrane to early endosomes, however with FTY720, internalized receptors fail to recycle back to plasma membranes (Oo, Thangada et al. 2007; Mullershausen, Zecri et al. 2009; Choi, Gardell et al. 2011). Ligand-binding properties dictating receptor fate serve as the basis for differentiating agonists from functional antagonists (Mullershausen, Zecri et al. 2009; Choi, Gardell et al. 2011). Yet in the context of functional antagonism, the bulk of our understanding for FTY720 and S1PR biology stems from studies on S1P1R activation, as FTY720 is shown to have greatest affinity for S1P1R (Brinkmann, Davis et al. 2002). Oo et al. (2007) suggested that the bifurcation in receptor fate by FTY720 (intracellular retention) compared to S1P (recycling) is in part responsible for its effects on immunosuppression.

5.6. Mechanisms of FTY720 function: agonist vs. antagonist

Two different (but perhaps not mutually exclusive) hypotheses have been proposed to explain the mechanism(s) by which FTY720 functions on CNS tissues. These hypotheses debate FTY720's modes of action following initial ligand-receptor ligation. First, is the notion that FTY720 signal transduction persists despite the absence of active S1PR agonism, while the alternative hypothesis argues that the absence of S1PR itself results in neuroprotection.

Agonist functions: FTY720 generates active tonic signals: In support of the former hypothesis, Mullershausen et al. (2009) found persistent cell activation by internalized S1PRs even in the absence of extracellular agonists. Comparing different S1P1R agonists, they found the persisted signaling to be specific to FTY720, and suggested that this differential agonist activity is an important feature of FTY720's efficacy in disease treatment (Mullershausen, Zecri et al. 2009). Following receptor internalization by FTY720, Mullershausen et al. (2009) showed that there was retention of active signaling inside the cell and suggested that the distinct FTY720-S1PR-binding conformation targets internalized receptors for differential sorting (Mullershausen, Zecri et al. 2009).

Antagonist functions: FTY720 removes S1PR from plasma membranes: As noted above, FTY720 antagonizes S1PR via permanent internalization rather than recycling to plasma membranes (as seen with pure S1PR agonists activation); the alternative hypothesis argues that the efficacy of FTY720 in reducing MS disease activity is mediated by the

physical removal of S1P1R itself. Choi et al. (2011) compared attenuation of S1P signaling via genetic ablation of S1P1R with FTY720 treatment, and in both conditions, disease activity was reduced. Their genetic approach allows for the deduction that neither the initial nor persistent S1PR activation served as the main effector mechanism mediating their observed responses (decreased EAE disease accompanied by reductions in astrogliosis, cytokine expressions and S1P concentrations (Choi, Gardell et al. 2011). They found that additional administration of FTY720 had no further effects in their transgenic mice lacking S1P1R on astrocytes, suggesting that the efficacy of FTY720 is through antagonizing astrocytic S1P1R (Choi, Gardell et al. 2011). Furthermore, studies on endosome trafficking and receptor fates have reported receptor polyubiquitination following FTY720 activation, suggesting that internalized receptors are targeted for degradation thus favoring signal termination rather than persistent activation (Oo, Thangada et al. 2007). Another plausible effect underscoring FTY720's influence on astrocytes may be the shift in the proportions of S1PR-subtypes activated by external stimuli. Specifically for FTY720 which binds (thus would removes) all S1PR except S1P2R, preincubatings cells with FTY720 would confer a signaling profile predominantly mediated by S1P2R. While over-expression studies have defined relevant characteristics unique to S1P2R activation, this model fails to recapitulate the dynamic shift from multi-receptor subtype activation to an S1P2R-dominated profile. Experimental approaches to address this hypothesis would need to depend on endogenous S1PR expressions and employ specific agonists binding exclusively to S1P2R.

5.7 FTY720 and S1P in MS

Common to many cell types, agonism by FTY720 is marked by the initial binding with surface S1PR, and the duration of agonism is assumed from there onwards until signal termination by ubiquitin-targeted receptor degradation (Pyne and Pyne 2002; Oo, Thangada et al. 2007). In contrast, activation via S1P is complete when receptors dissociate from its ligand-complexed form and return to plasma membranes (Pyne and Pyne 2002). A notable end-point difference therefore, is the reduction in surface S1PR following FTY720 treatment but not S1P. This serves as the main corollary in explaining their opposing effects on MS pathogenesis, with S1P proposed to be disease-inductive and FTY720 alleviating neuroinflammation (Van Doorn, Van Horssen et al. 2010; Choi, Gardell et al. 2011; Kipp and Amor 2012). In vitro studies show that S1P can promote cellular responses in astrocytes predicted to promote MS pathology, and emerging data indicate that modulating the S1PR signaling axis will impact on mechanisms contributing to MS pathogenesis (Pebay, Toutant et al. 2001; Mullershausen, Craveiro et al. 2007; Kimura, Ohmori et al. 2008; Choi, Gardell et al. 2011). S1PR activation by its endogenous ligand is proposed to be a mediator of astrogliosis as S1P is shown to induce significant astrocyte proliferation in an ERK1/2 activation-dependent manner (Sorensen, Nicole et al. 2003; Osinde, Mullershausen et al. 2007). Sorensen et al. (2003) reported that direct in vivo injections of S1P into the striatum of 90-day-old mice resulted in significant astrogliosis. Clinically, increased S1P levels in cerebral spinal fluids of MS patients are observed, and S1PRs are upregulated on reactive astrocytes in MS lesion borders, further suggesting the involvement of S1P signaling in chronic CNS

inflammation and MS pathogenesis (Dev, Mullershausen et al. 2008; Kulakowska, Zendzian-Piotrowska et al. 2010; Fischer, Alliod et al. 2011). Since astrocytes are major targets for S1P signaling in the CNS, the mechanism by which FTY720 functions and/or interferes with this response is the main focus of this thesis.

Hypotheses & Objectives

Our goals were to identify neuroinflammation-relevant responses in primary human astrocytes to FTY720. We examined the dynamics of FTY720 signaling, and correlated functional outcomes to drug-dosing regimens. We hypothesize that FTY720 imposes an anti-inflammatory influence on human fetal astrocytes. First we examined astrocyte signaling to FTY720 via Western blot, and tested FTY720's potential to desensitize S1PR activation. We went on to determine the duration of signal desensitization and modeled the clinical use of this drug by examining the effects of daily-repeated FTY720 exposures on astrocytes. Our functional aims were to test the effects of FTY720 on astrogliosis by measuring astrocyte proliferation, and also whether pretreatment of FTY720 will affect astrocyte responses to activating signals from proinflammatory cytokines such as IL-1β.

Given the novelty of FTY720, unraveling astrocytes' responses to this drug will extend our understanding for this therapeutic intervention. Furthermore, exploring the functional consequences of S1PR signaling on astrocytes, and how this is impacted by FTY720 treatment will propel us in developing assays that could have direct relevance to MS tissues. Since MS is marked by an associated activation of astrocytes, and increasing studies show the importance of S1PR signaling in MS pathogenesis, exploring how FTY720 affects this endogenous glial cell population is important to the overall understanding of MS pathobiology.

Materials & Methods

7.1 Human fetal astrocyte isolation and cell culture

Central nervous system tissues were obtained from the human fetal tissue repository at Albert Einstein College of Medicine, Bronx, NY. Experiments were carried out with guidelines approved by McGill University and the Canadian Institutes for Health Research. Cells were isolated as previously described in (Williams, Dooley et al. 1995). Briefly, fetal brain tissue (gestational age 15–18 weeks) was minced and digested with DNase (Roche) and trypsin (Invitrogen). The tissue was then dissociated through a nylon mesh and the flow through was plated at 10^6 cells/mL in high glucose DMEM supplemented with 10% fetal calf serum (FCS) (v/v), penicillin/streptomycin and glutamine (Invitrogen). Cells were grown in a humidified incubator maintained at 37^{o}C, 5% CO_2 and passaged every 14 days. All experiments were conducted on the 3rd or 4th passage. For serum-free assays, astrocyte media was changed to Dulbeco's defined medium supplemented with 1% bovine serum albumin (BSA), penicillin/streptomycin and N1 factor (Gibco Life Technologies).

7.2 Pharmaceutical compounds

FTY720

In all of the experiments, the phosphorylated form of FTY720 was used (provided by Novartis, Basel, Switzerland). FTY720 powder was reconstituted in dimethyl sulfoxide, HCl 50 mM, aliquotted and stored in -20^{o}C until use. In all of the experiments, a final concentration of 100nM was used to treat the cells.

Sphingosine-1-phosphate

Sphingosine-1-phosphate was purchased from Sigma and S1P powder was dissolved in 100% methanol, aliquotted and stored in -20^{o}C until use. In all of the experiments, a final concentration of 100nM was used to treat the cells.

7.3 S1PR mRNA expression studies

Astrocytes were seeded on 6 well-plates and cultured in defined medium. For basal S1PR studies, RNA was collected when astrocytes reached 80% confluency. For the overnight FTY720-S1P1R experiments, astrocytes were treated with FTY720 (or vehicle) for 18 hours before collecting the RNA. The extended FTY720-S1P1R expression studies were performed by either treating astrocytes once at the onset of experimentation (1x) or daily for 3 days (3x) with FTY720 or vehicle. In all experiments, astrocytes were lysed with RTL buffer (Qiagen) supplemented with β-mercaptoethanol, and total RNA was extracted with the Mini-elute Qiagen RNeasy mini kit following manufacture's instructions. Samples were treated with DNase (Qiagen) and 2ug of RNA were reverse transcribed into cDNA using random hexaprimers (Roche) and the Moloney murine leukemia virus-RT enzyme (Invitrogen) in a thermocycr at 42°C, 75°C for 60 minutes, 4°C for 10 minutes. Expression of genes encoding S1P1R, S1P3R, S1P5R and the endogenous house-keeping gene 18S, were determined by real-time PCR using TaqMan probes and Assays-on-Demand primers (Applied Biosystems). Transcript levels were measured using the ABI Prism 7000 sequence detection system (Applied Biosystems) with default temperature settings (2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles

of 15 seconds at 95°C, 1 minute at 60°C). Analysis of the house-keeping gene 18S showed no significant difference between replicates, thus the averaged expressions were used to normalize values from genes of interest. Relative levels of S1P1, 3, 5R mRNAs were expressed as arbitrary units derived from calculating the difference between the S1PR and 18S cycle values. All receptor studies are reported in cycle thresholds (**Δ**CT) values and transcripts abundance were classified into discrete ranges described in (Durafourt, Lambert et al. 2011), where **Δ**CT 5–10, high expression; 10<**Δ**CT≤15, intermediate expression; 15<**Δ**CT≤20, low expression; and **Δ**CT<20, defined as rarely detected.

7.4 S1PR-activated ERK phosphorylation studies

In all of the ERK signaling studies, astrocytes were seeded on 6 well-plates and cultured to 80% confluency before experimentation.

Acute ligand exposure and pERK activation

For acute ligand exposure, astrocytes were serum-starved for 6 hours and then treated with FTY720 (or S1P) for 15 min.

FTY720-mediated pERK signal desensitization

To determine whether FTY720 desensitizes signaling pathways in astrocytes,

FTY720 (or S1P) was given overnight before re-exposing astrocytes to FTY720 (15 min).

Recovery of astrocyte pERK signaling following FTY720 treatment

Recovery of signaling responses were assessed by subjecting astrocytes to an initial dose of FTY720 (without washout) and culturing for 72 hours before re-exposing cells to FTY720 (15 min).

pERK activation by serum

As a control for the specificity of S1PR-related pERK desensitization, astrocytes were treated with FTY720 at the onset of experimentation (1x) without washout or daily for 3 days (3x), followed by serum (10% fetal calf serum in DMEM) for 15 mins.

Sustained pERK desensitization by repeated FTY720 administrations

Astrocytes were given FTY720 once (with- or without washout after 1 hour), or daily for 5 days. Following treatments, cells were activated by FTY720 for 15 mins before collecting cell lysates.

In all signaling studies, astrocytes were washed with phosphate-buffered saline (PBS) before collecting whole-cell lysates in 200uL of RIPA buffer (1% SDS, 1% deoxycholate acid, 1% Igepal (NP-40), 150 mM NaCl, 50mMTris) with Baculogold protease inhibitor (BD Biosciences) and phosphatase inhibitor (1 mM Na_3VO_4, Sigma). All samples were stored in -80^{o}C until use.

Western blotting

Polyvinylidene difluoride membranes were immunoblotted with anti-phospho-ERK1/2 antibody (monoclonal rabbit anti-mouse antibodies 1:1000; Cell Signaling Technology) and detected with horseradish peroxidase-conjugated secondary antibodies (1:1000; Calbiochem) and ECL Plus reagents (GE Healthcare). Membranes were then stripped with Reblot (Millipore) and reprobed for loading control (monoclonal anti-β-actin 1:1000; Invitrogen). Protein band intensities were quantified using ImageJ software (National Institutes of Health) and reported data are expressed as relative fold-change to untreated controls normalized to 1.

7.5 Proliferation Assays

$5x10^4$ cells were plated on a 48-well plate for 24 hours before serum-starvation. In the overnight treatment studies, astrocytes were given S1P or FTY720 for 18 hours before fixation. For the FTY720-pretreatment experiments, astrocytes were treated for 24 hours with S1P or FTY720 on Day 0. Agents and media were then aspirated and cells were washed 3x with PBS, and astrocytes were treated for another 24 hours with S1P (Day 1). Cells were fixed with 4% paraformaldehyde and permeated with 0.25% Triton-X100 (Sigma). To determine the proportion of astrocytes undergoing mitotic cell division, astrocytes were stained with anti-Ki67 antibody (rabbit monoclonal FITC-conjugated 1:100; Abcam) and cell nuclei dye (Hoechst 1:10000; Invitrogen, Molecular Probes). Images were taken from 16 fields per well and analyzed by MetaXpress (Molecular Devices) cellular imaging analysis software (cell-scoring module). Quantitative output measures by MetaXpress processing were used to calculate proliferation rate indices,

where proportion of astrocyte positive for Ki67 was determined as a function of total cell number per field (Hoechst).

7.6 Ratiometric measurements of intracellular calcium induced by interleukin-1βeta activation

Astrocytes were plated at an initial concentration of $2x10^5$ cells/ml in 96-well (flat bottom) plates and cultured in serum-free media. FTY720 treatment(s) were either given once on day 1 (1x) or daily for 5 days (5x). Following treatments, astrocytes were washed with PBS and subsequently loaded with a calcium-sensitive fluorescent dye, fura-2 AM (5 uM, Molecular Probes) suspended in calcium-free Ringer's solution (130mM NaCl, 3mM KCl, 1mM $MgCl_2$, 10mM HEPES, pH adjusted to 7.4) for 45 min in a humidified chamber at 37°C, 5% CO_2. Cells were washed 2x with PBS to remove extracellular fura-2AM and were incubated in Ringer's solution for an additional 30 min at room temperature for complete hydrolysis of acetoxymethyl ester before imaging. The Wallac Victor^3 (Perkin-Elmer) fluorescent microplate reader was used to measure ratiometric intracellular calcium concentrations and 10ng/ml IL-1β (recombinant human IL-1 beta, PepproTech) suspended in PBS was loaded in the integrated injector, delivered at a volume of 25ul/well. To establish baseline values, 5 measurements were taken before IL-1β injection and 30 consecutive recordings immediately following IL-1β delivery into each well. Data reported represent means from 3 independent experiments with each observation averaged over 8 wells per condition. Fluorescence emission ratios are expressed as intracellular calcium values using the formula described in Grynkiewicz et

al. (1985) and the magnitudes of response (to IL-1β agonist) are calculated by taking the difference between peaks of calcium curves with baseline values.

7.7 Cytokine Production

Astrocyte supernatants were generated from cells treated with either a single dose (1x) or daily FTY720 for 3 days (3x). Following FTY720 treatment(s), astrocytes were stimulated with IL-1β for 24 hours before collecting supernatants. The levels of cytokines (IL-6 and IP-10) present in supernatants were measured in duplicates using ELISA plates following manufacturer's guide (BD Biosciences).

Statistical analyses

Statistical analyses were performed with Prism 5 (GraphPad Software). One-way analysis of variance (ANOVA) with Bonferroni post hoc tests were used to compare mean values. Significance was accepted at the $p<0.05$ level, and the number of individual studies performed for each set of experiments is indicated in the results and figure legends. The following notation was adopted for figure representation: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Results

8.1 S1P receptor expression

As shown in Figure 1a, human fetal astrocytes express S1PRs 1, 3, but low levels of S1P5R mRNA. Exposure of the cells to FTY720 over 24-hours (Figure 1b) or 72-hour (Figure 1c) time periods did not produce significant changes in receptor S1P1R mRNA expression.

8.2 S1P receptor desensitization studies

To address the issue of S1PR desensitization in fetal human astrocytes, we gave astrocytes an initial exposure to FTY720 (compared with the natural ligand, S1P), and measured both pERK activation and proliferation responses to subsequent ligand challenge.

8.2.1. pERK responses

Exposing astrocytes that have been kept in serum free medium for 24 hours to FTY720 (or S1P) for 15 minutes induced pERK (Figure 2a). This brief time period of stimulation was selected based on initial time response curves reported in (Durafourt, Lambert et al. 2011), and is similar to that used by (Mullershausen, Zecri et al. 2009) in their studies with rodent astrocytes. When astrocytes were maintained for 24 hours in presence of an initial dose of FTY720 (models acute daily use of drug), pERK response at 24 hours was

reduced compared to cells maintained in culture medium alone or with added vehicle control (Figure 2b). This desensitization response was not observed in cells cultured with S1P for 24 hours (Figure 2c), as pERK responses are comparable to control cells. Figure 2d shows recovery from FTY720-mediated pERK desensitization by 72 hours.

8.3 Proliferation

Figure 3a shows immunohistochemistry stainings for cells undergoing cell division (Ki67-positive). Measuring the proportion of cells incorporating Ki67, we detected an increase in astrocyte proliferation that is significantly beyond basal rate when given the natural ligand S1P but not FTY720 (Figure 3b). For subsequent proliferation studies, we used S1P to induce the response. Astrocytes cultured in the presence of FTY720 overnight no longer showed a proliferative response when exposed to S1P for a subsequent 24 hours (shown in Figure 3c). Cells maintained in culture alone or in the presence of S1P proliferated in response to subsequent S1P challenge.

8.4 Extended treatment studies on pERK signaling

Astrocytes exposed to FTY720 daily for 5 days (Figure 4) or 3 days (Figure 5) and re-exposed with FTY720 showed a loss of pERK compared to astrocytes maintained in culture medium alone or in presence of FTY720 added at time of culture initiation (either left in (1x) or removed after one hour (1hr)). Addition of serum to astrocyte cultures

treated repeatedly with FTY720 provoked a robust pERK response (Figure 6) indicating the integrity of this signaling pathway was not affected by FTY720.

8.5 IL-1β-dependent calcium mobilization

As illustrated in Figure 7a, IL-1β induces calcium mobilization from astrocytes, as expected. Repeated daily dosages of FTY720 resulted in the blunting of the IL-1β-evoked calcium response when compared to effects of vehicle alone (Figure 7a and b). There was no inhibition in calcium release after 5 days in astrocyte cultures exposed to a single dose of FTY720 from outset. At the initial overnight time point following exposure to FTY720, there was an apparent partial inhibition of calcium response by IL-1β (Figure 8). Neither FTY720 nor S1P themselves induced significant calcium release from astrocytes when compared to their vehicles (Figure 9a and b) (changes from baseline: FTY720: 468±SEM 193nM vs. vehicle: 942±SEM 434nM; S1P: 463±SEM 171nM versus vehicle: 443±SEM 94 nM, n=5 separate experiments).

8.6 Cytokine secretion

FTY720 repeatedly for 3 days did not significantly block IL-1β's induction of cytokines/chemokines measured using IL-6 or IP-10 (Figure 10a and 10b, respectively).

Discussion

FTY720 is shown to reduce activities relevant to neuroinflammation by acting through astrocytes' S1PR, however the mechanisms describing this observation are debated. The importance of S1PR signaling in astrocytes led us to investigate the unique properties of FTY720, functioning as an antagonist via S1PR internalization or a persisted agonist. Furthermore, we investigated the effects of repeated dosing of FTY720 on astrocyte responses pertaining to neuroinflammation.

FTY720 binds S1PRs on astrocytes and activate a number of signaling axes. Human fetal astrocytes express abundant levels of S1P1R and S1P3R (but very low S1P5R), suggesting that they can constitutively respond to S1P-related ligands. Astrocytes respond to FTY720 ligation in a similar manner to initial agonism by S1P in that they both activate MAPK signaling cascades.

To begin describing FTY720's antagonism, we validated the lack of surface S1PR by showing that overnight incubation with FTY720 desensitized pERK signaling to re-stimulation by S1PR ligands. Functionally, astrocytes exposed to FTY720 did not respond to subsequent S1P by proliferation, suggesting that FTY720 effectively removed S1PRs off of the cell's surface. FTY720 may, in consequence, illicit anti-inflammatory influences on astrocytes by blocking S1P-mediated mitogenesis. Endogenously, S1P signals astrocytes to proliferate and Sorensen et al. (2003) demonstrated that local S1P injections in the CNS resulted in astrogliosis. The removal of surface S1PR by FTY720 renders astrocytes unresponsive to external S1P cues, thus serving as a potential target for minimizing pathological glial proliferation and reactive gliosis development. Moreover,

we extended this observation by determining the duration of FTY720-induced desensitization, and found that pERK signaling approaches baseline by 72 hours (3 days). This implies that the elements in the pERK pathway have recovered, including the return of S1PR to plasma membranes. We found no evidence of de novo synthesis of S1P1R based on levels of mRNA expression; we speculate that S1PRs are reconstituted to plasma membranes from the dissociation of S1PR-ligand complex.

We note, however, FTY720's influences on astrocytes are specific to given functions, as the astrocytes' capacity to secrete cytokines (IL-1, IP-10) upon IL-1β activation was not affected by FTY720 treatments. In line with this observation, the induction of pERK by serum was robust, despite the signal desensitization when stimulated by FTY720. This supports the loss of S1PR agonism when preceded with FTY720 treatment, and not due to a defect in cells' innate ability to signal through pERK.

To reflect the clinical use of FTY720, we examined the effects of daily-repeated drug administrations. We found that the desensitized pERK response was maintained throughout the course of treatment (3 or 5 days). While a clear consequence of surface S1PR removal would be attenuating receptor-activation by external stimuli, we wanted to know whether daily FTY720 treatment would have non-S1PR-related functions. As such, we were prompted to investigate the effects of FTY720 treatment on cytokine activation through the IL-1β receptor-signaling pathway. IL-1β is a potent agonist for calcium signaling in astrocytes and the mechanism by which exogenous IL-1β evokes astrocyte calcium rises is reported to be via PLC in the NFkB pathway. We found that FTY720 exposure on astrocytes dampened calcium responses to IL-1β activation, and chronic FTY720 applications completely inhibited the calcium rises. In the context of MS

pathogenesis, FTY720 treatments may render astrocytes less prone to inflammatory activations by signaling through calcium-dependent pathways. There are no data to suggest that lack of S1PR (per se) should affect IL-1β's capacity to illicit intracellular calcium release and as such, we propose that the attenuation in calcium response is mediated by continuous FTY720 function. The mechanism underlying the observed phenomena may be best explained by zu Heringdorf et al. (2003)'s work on S1PR subtypes and calcium signaling pathways. Results from Meyer et al. (2004) indicate that the capacity of S1P to activate calcium-signaling pathways is limited to specific S1P receptor activation. Intracellular calcium signaling may be regulated by the patterns of S1PR engagement, as differential coupling of S1PR subtypes to G proteins interact in response to agonist-activation. Our receptor expression data shows the constitutive co-expression of S1P1R and S1P3R in our human fetal astrocyte cultures, and Meyer et al. (2004) amongst others comparing the effectiveness by which S1PR couple calcium revealed that S1P3R is the primary receptor subtype activating calcium-stores (Meyer Zu Heringdorf 2004; Giussani, Ferraretto et al. 2007). zu Heringdorf et al. (2003) showed that S1P1R signaling inhibits calcium transients evoked by other receptors through protein kinase C, and FTY720 is known to preferentially bind S1P1R. Our results reflect this notion as we show that S1PR engagement with FTY720 attenuated calcium responses to IL-1β activation. Collectively, our data parallels their observations where pre-activation of S1P1R inhibits agonist-evoked calcium mobilization (zu Heringdorf, Vincent et al. 2003).

Of note, only the effects observed with genetically ablating S1PR are purely attributable to S1PR antagonism; surface receptor removal by pharmaceutical agents

(FTY720 specifically) cannot exclude the potential for downstream signal activation by the internalized complex. Notably, the initial receptor-FTY720 engagement triggers a cascade of events, which are predicted to be very different than S1P-mediated downstream responses. As such, examining responses exclusively to the lack of S1PR may overlook the contributions brought about by FTY720 intracellularly. Undoubtedly, our data also cannot exclude the potential for signal activation initiated by the lack of surface S1P receptors itself, but a more plausible explanation for FTY720's involvement in suppressing calcium-efflux by exogenous agonists is the aforementioned persisted activation of a negative calcium regulator. FTY720-engaged S1P1R maintains calcium suppression in a similar (but opposite) fashion to FTY720's effect on pERK signal desensitization, with the former acting as an agonist and the latter functioning as a receptor antagonist.

Collectively, our in vitro studies indicate that daily FTY720 exposures can regulate neuroinflammation-relevant responses in astrocytes by acting as a functional antagonist for external stimuli (natural ligand S1P), while sustaining internalized receptor-dependent functions (inhibit IL-1β evoked calcium mobilization). Instead of the conventional description of temporal shifts from S1PR agonism to antagonism, we discovered that these seemingly opposing functions occur in tandem. Furthermore, we show that the effects of FTY720 on human astrocytes are dependent on the dosing regimen.

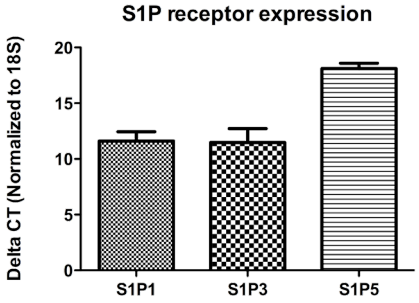
Conclusions

FTY720's clinical use as a daily oral therapy for MS demonstrates efficacy in reducing disease activity, and the identification of cognate S1PR binding has opened research avenues for understanding the involvement of sphingolipid signaling in MS pathophysiology. Beyond its effect at the periphery, emerging studies suggest a role for FTY720 in exerting anti-inflammatory influences on CNS cells relevant to MS. Astrocytes, in particular, abundantly expressing S1PRs 1 and 3 are shown to respond to FTY720 by modulating cellular responses resulting in reduced astrogliosis. Our aims were to identify signaling and functional responses relevant to neuroinflammation in primary human astrocytes, and how the FTY720 dosing regimen impacts these effects. While we observed FTY720's antagonistic effects on S1PR, we also identified functional changes in astrocytes that are indicative of prolonged intracellular FTY720 function. We show that FTY720 blocks S1PR reactivation by external stimuli (FTY720, pERK activation; S1P, proliferation), and interferes with IL-1β activation via suppressing calcium mobilization in human astrocytes. Our observations expand on the current understandings for S1P1R activation and provide additional evidence for FTY720's dualistic agonist-antagonist effects. We report that the inhibitory actions of chronic (repeated) FTY720 exposures on calcium efflux, accompanied by desensitization in pERK signaling, demonstrate the dual agonist and antagonist effects by FTY720. Collectively, we addressed the 'paradoxical' functions of FTY720 by suggesting that these antagonist and agonistic effects occur in tandem. Dosing strategies using FTY720 and other S1PR-modulators may need to consider the cell type and function(s) being

targeted. In the context of astrocytes, diverse functional responses mediated by S1PRs may contribute to promoting or inhibiting the severity of CNS-directed inflammatory disorders.

Figures

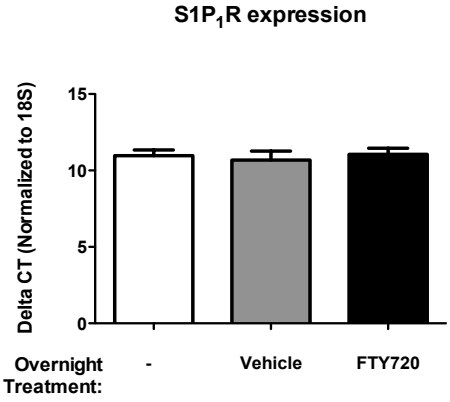
FIGURE 1a



S1P receptor expression by human fetal astrocytes.

Reverse transcriptase-polymerase chain reaction (RT-PCR) probing for S1P1, 3, 5 receptor expressions. Figure showing the relative level of S1P receptor transcripts expressed by human fetal astrocytes (normalized to 18S housekeeping gene). Astrocytes express abundant S1P1R and S1P3R, but low S1P5R (n= 4).

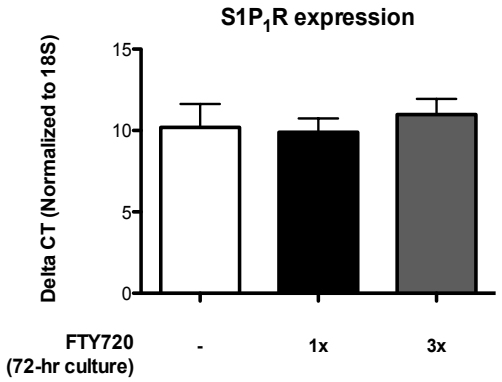
FIGURE 1b



FTY720 and S1P1R expression by human fetal astrocytes.

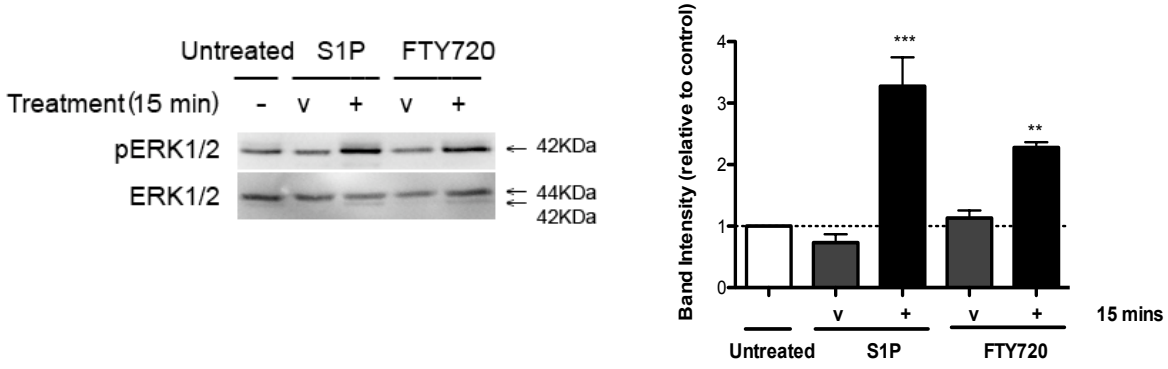
Human fetal astrocytes treated overnight (18 hours) with FTY720 or vehicle. S1P1R expressions were not altered by FTY720 exposure (delta CT normalized to 18S housekeeping gene, n=3).

FIGURE 1c



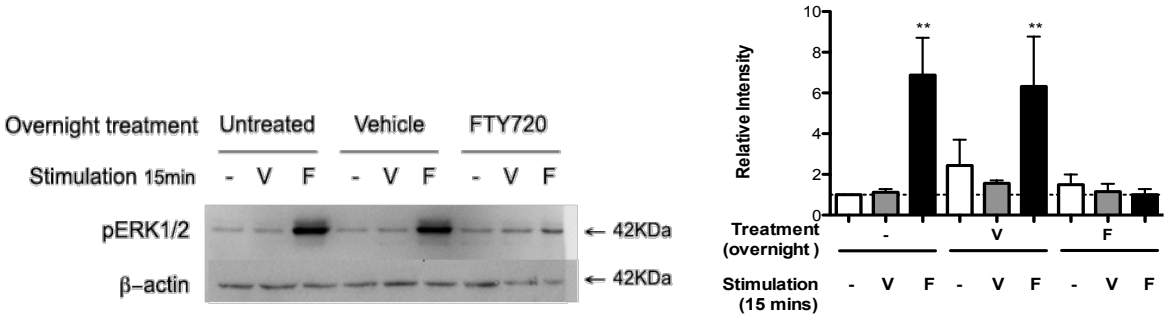
Chronic FTY720 did not change S1P1R expressions in human fetal astrocytes.
Astrocytes cultured for 72 hours with FTY720 given once (1x) at onset or daily (3x). S1P1R expressions are not significantly altered by FTY720 treatment(s). S1P1R levels normalized to 18S housekeeping gene, n=3.

FIGURE 2a



Human fetal astrocytes acutely exposed to FTY720 activated pERK
Western blot probing for pERK (left). Lane 1 represents untreated control and lane 2 is MeOH vehicle control for S1P 100nM (lanes 3). Lanes 4 and 5 are DMSO-HCl (50mM) vehicle control and FTY720 100nM, respectively. Bottom bands represent total ERK loading control. Quantified band intensity relative to untreated control, n=3 (right).

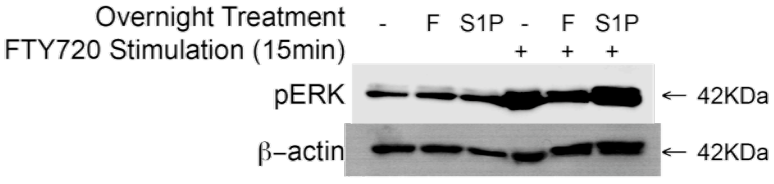
FIGURE 2b



FTY720 treatment (overnight) desensitized subsequent pERK activation by acute FTY720 exposure in human fetal astrocytes.

Astrocytes treated overnight with FTY720 followed by re-exposure to the drug. FTY720 induced significant ERK phosphorylation in untreated astrocytes and in astrocytes pretreated overnight with vehicle (left). Brief exposure to vehicle did not activate pERK, and pre-exosing astrocytes to FTY720 resulted in a blunted pERK signal when activated with FTY720 for 15 mins. Quantified band intensity relative to untreated control, n=3 (right).

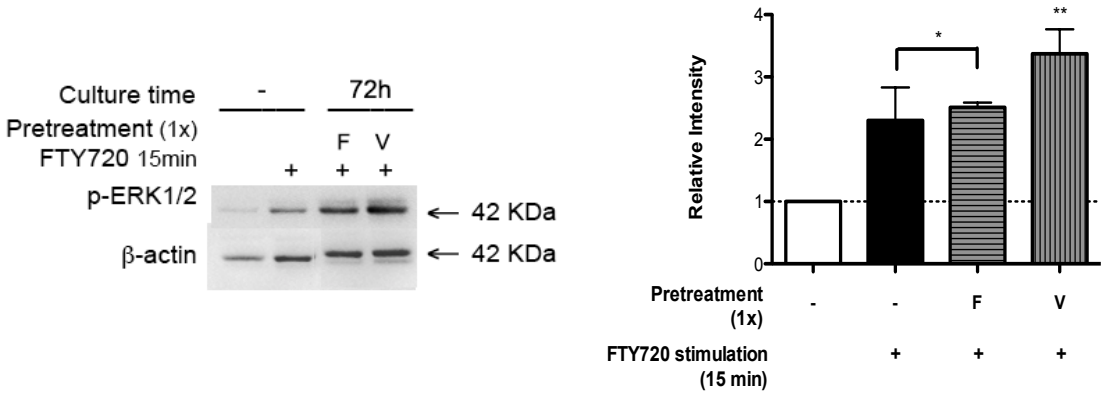
FIGURE 2c



S1P pretreatment did not result in a desensitization response to subsequent acute FTY720 challenge.

Western blot showing that astrocytes pretreated with 100nM S1P did not result in a blunted pERK activation when given FTY720 for 15 mins.

FIGURE 2d

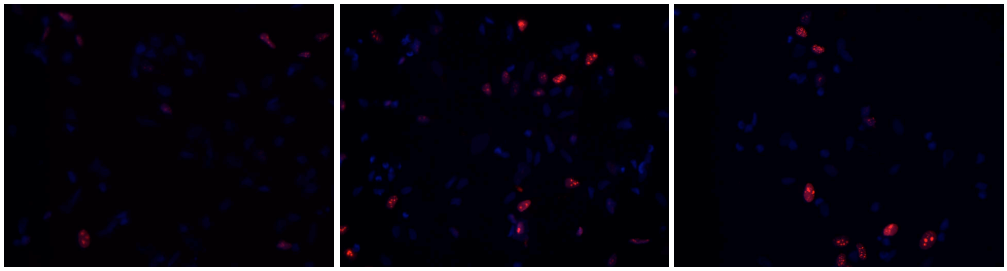


Astrocyte pERK resensitization from FTY720 treatment recovers by 72 hours

Astrocytes re-activated with FTY720 (15mins) 72 hours following initial FTY720 treatment. Western blot showing the recovery from FTY720-mediated pERK desensitization at 72 hours (left). Quantified relative band intensity relative to untreated control, n=3 (right).

FIGURE 3a

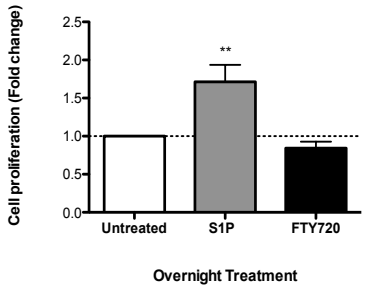
i. **ii.** **iii.**



S1P receptor agonists and astrocyte proliferation.

Immunohistochemistry stainings for cells undergoing cell division, Ki67-positive (red) and total cell nuclei (blue). i. Untreated standard (basal proliferation). ii. S1P overnight. iii. FTY720 overnight.

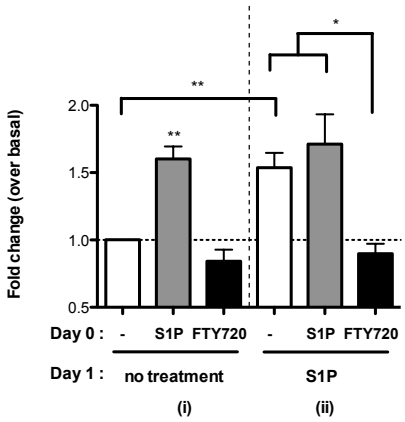
FIGURE 3b



S1P increased astrocyte proliferation, FTY720 did not

Proliferation index determined as the proportion of astrocytes undergoing proliferation (Ki67+) with respect to population (Hoechst nuclear stain). Fold change reported as a relative fraction to basal rate of proliferation. S1P induced a 1.8-fold increase in astrocyte proliferation whereas FTY720 did not induce proliferation beyond basal rates, n=3.

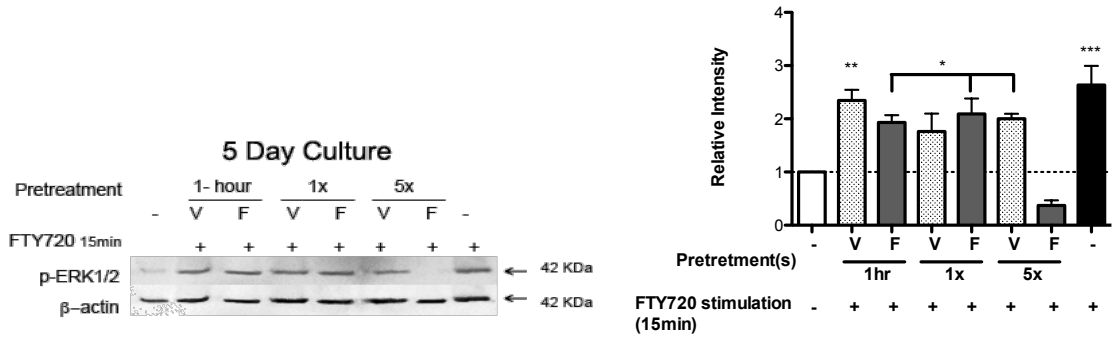
FIGURE 3c



FTY720 pre-incubation blocked astrocytes' proliferative response to S1P.

Astrocytes pretreated with FTY720 or S1P (Day 0), and subsequently incubated with S1P for another 24 hours (Day 1). Day 0 S1P exposure increased astrocyte proliferation whereas FTY720 was comparable to the basal rate. Day 1 S1P exposure induced mitogenesis in untreated control and S1P-treated astrocytes, but not in the FTY720-pretreated group, n=3.

FIGURE 4



Repeated (daily) FTY720 administrations maintained pERK desensitization.

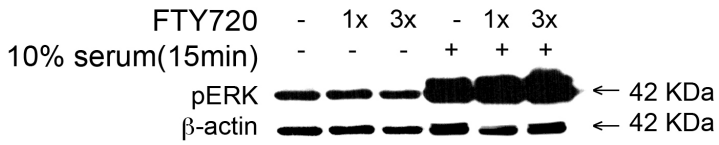
Western blot showing desensitization of pERK signaling in response to a single dose of FTY720 with or without washout after 1 hour, or repeated FTY720 dosing for 5 days (left). pERK activation by FTY720 was observed in the short (1hr) and single dose (1x) FTY720 conditions but not in the repeated daily FTY720 condition (5x). Quantified relative band intensity relative to untreated control, n=3 (right).

FIGURE 5

Repeated (daily) FTY720 administrations maintained pERK desensitization.

Western blot showing desensitization of pERK signaling in response to a single dose of FTY720 with or without washout after 1 hours, or repeated FTY720 dosing for 72 hours. pERK activation by FTY720 was observed in the short (1-hour) and single dose (1x) FTY720 conditions but not in the repeated daily FTY720 condition (3x).

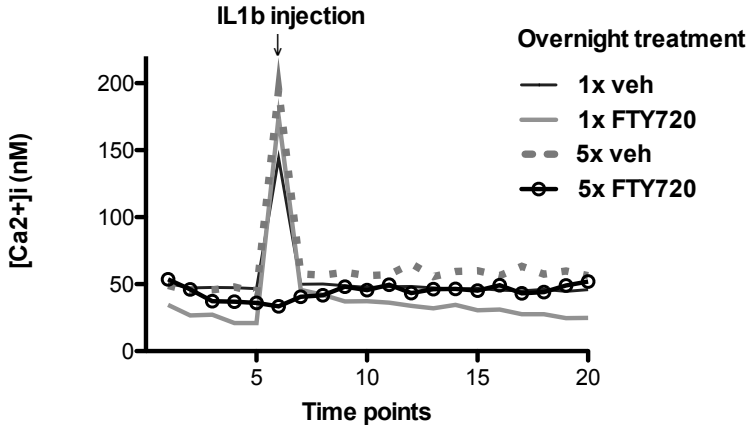
FIGURE 6



FTY720 exposure(s) did not inhibit pERK signaling by nonS1PR agonists (fetal calf serum, FCS).

Western blot showing astrocytes treated with a single dose, or repeated daily for 3 days with FTY720. In both conditions FTY720 treatment did not desensitize pERK signaling in response to FCS activation (10% FCS in DMEM).

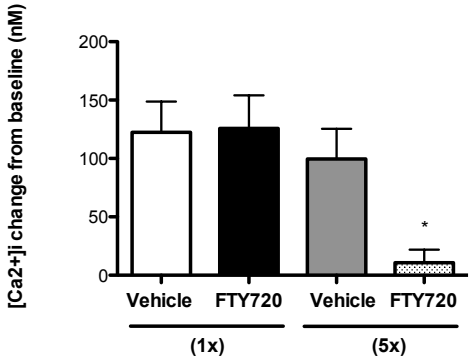
FIGURE 7a



Repeated FTY720 exposures diminished astrocytes' response to IL-1β by Ca^{2+} mobilization.

Representative traces showing IL-1β applied to astrocytes treated once (1x) without wash-out or daily (5x) FTY720. IL-1β evoked Ca^{2+} efflux in astrocytes treated once with FTY720 (1x) whereas in astrocytes treated daily with FTY720 (5x), the Ca^{2+} response was inhibited.

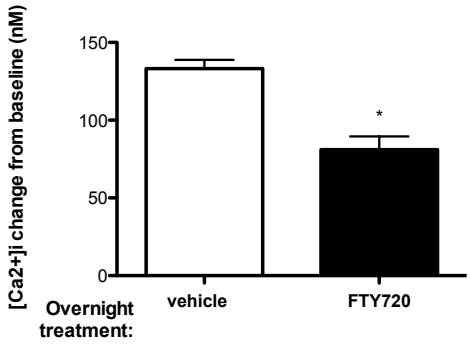
FIGURE 7b



Repeated FTY720 administration diminished astrocytes' response to IL-1β by Ca^{2+} mobilization.

Bar-graph showing the SEMs from 3 independent experiments. IL-1β evoked Ca^{2+} efflux in astrocytes treated once with FTY720 (1x) whereas in astrocytes treated daily with FTY720 (5x), the Ca^{2+} response was inhibited.

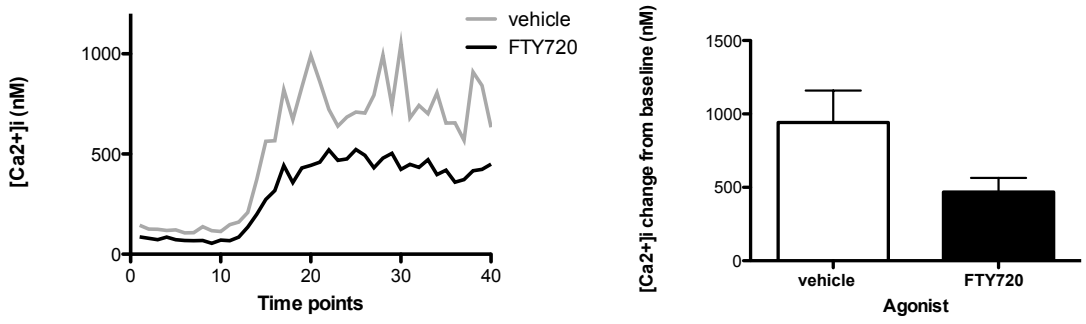
FIGURE 8



FTY720 exposure decreased astrocytes' response to IL-1β by Ca^{2+} mobilization.

Bar graph representing means from 3 independent experiments. Ca^{2+} mobilization was decreased in astrocytes pretreated overnight FTY720 (compared to vehicle pretreatment) upon IL-1β challenge.

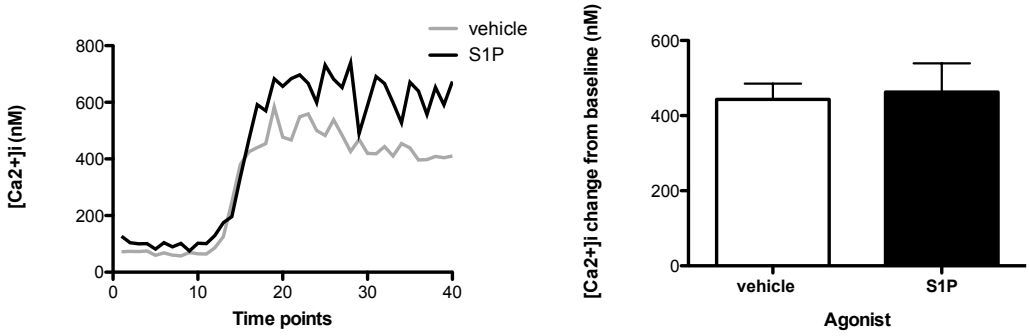
FIGURE 9a



Ca^{2+}evoked by acute FTY720 administration was lower than the effects of vehicle.

(Left) Representative traces showing the effects of FTY720 (and vehicle) on astrocyte Ca^{2+}mobilization. Vehicle increased intracellular Ca^{2+} concentrations in astrocytes greater than that observed with FTY720 application. (Right) quantified means of intracellular Ca^{2+} change from baseline.

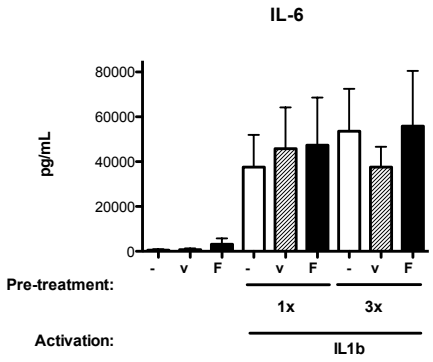
FIGURE 9b



S1P did not significantly increase Ca^{2+} mobilization in astrocytes relative to the effects of vehicle.

(Left) Representative traces showing the effects of S1P (and vehicle) on astrocyte Ca^{2+} mobilization. S1P did not significantly increase intracellular Ca^{2+} concentrations in astrocytes compared to its vehicle. (Right) quantified means of intracellular Ca^{2+} change from baseline.

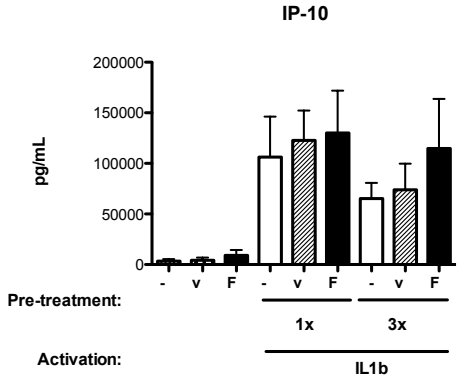
FIGURE 10a



FTY720 did not alter the levels of IL-6 secreted by astrocytes in response to IL-1β activation

Bar-graph showing the SEMs from 3 independent experiment. Astrocytes produce significant levels of IL-6 in response to IL-1β activation, and the levels of IL-6 produced were not affected by FTY720 treatment(s) (1x and 3x).

FIGURE 10b



FTY720 did not alter the level of IP-10 secreted by astrocytes in response to IL-1β activation

Bar-graph showing the SEMs from 3 independent experiment. Astrocytes produce significant levels of IP-10 in response to IL-1β activation, and the levels were not affected by FTY720 treatment(s) (1x and 3x).

References

Albert, R., K. Hinterding, et al. (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." Journal of medicinal chemistry **48**(16): 5373-5377.

Anada, Y., Y. Igarashi, et al. (2007). "The immunomodulator FTY720 is phosphorylated and released from platelets." European journal of pharmacology **568**(1-3): 106-111.

Anelli, V., R. Bassi, et al. (2005). "Extracellular release of newly synthesized sphingosine-1-phosphate by cerebellar granule cells and astrocytes." Journal of neurochemistry **92**(5): 1204-1215.

Bassi, R., V. Anelli, et al. (2006). "Sphingosine-1-phosphate is released by cerebellar astrocytes in response to bFGF and induces astrocyte proliferation through Gi-protein-coupled receptors." Glia **53**(6): 621-630.

Beskina, O., A. Miller, et al. (2007). "Mechanisms of interleukin-1beta-induced Ca2+ signals in mouse cortical astrocytes: roles of store- and receptor-operated Ca2+ entry." American journal of physiology. Cell physiology **293**(3): C1103-1111.

Biernacki, K., A. Prat, et al. (2001). "Regulation of Th1 and Th2 lymphocyte migration by human adult brain endothelial cells." Journal of neuropathology and experimental neurology **60**(12): 1127-1136.

Brinkmann, V. (2009). "FTY720 (fingolimod) in Multiple Sclerosis: therapeutic effects in the immune and the central nervous system." British journal of pharmacology **158**(5): 1173-1182.

Brinkmann, V., M. D. Davis, et al. (2002). "The immune modulator FTY720 targets sphingosine 1-phosphate receptors." The Journal of biological chemistry **277**(24): 21453-21457.

Chi, H. (2011). "Sphingosine-1-phosphate and immune regulation: trafficking and beyond." Trends in pharmacological sciences **32**(1): 16-24.

Choi, J. W., S. E. Gardell, et al. (2011). "FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation." Proceedings of the National Academy of Sciences of the United States of America **108**(2): 751-756.

Chun, J. and V. Brinkmann (2011). "A mechanistically novel, first oral therapy for multiple sclerosis: the development of fingolimod (FTY720, Gilenya)." Discovery medicine **12**(64): 213-228.

Chun, J. and H. P. Hartung (2010). "Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis." Clinical neuropharmacology **33**(2): 91-101.

Dal Pra, I., A. Chiarini, et al. (2005). "Roles of Ca2+ and the Ca2+-sensing receptor (CASR) in the expression of inducible NOS (nitric oxide synthase)-2 and its BH4 (tetrahydrobiopterin)-dependent activation in cytokine-stimulated adult human astrocytes." Journal of cellular biochemistry **96**(2): 428-438.

Dev, K. K., F. Mullershausen, et al. (2008). "Brain sphingosine-1-phosphate receptors: implication for FTY720 in the treatment of multiple sclerosis." Pharmacology & therapeutics **117**(1): 77-93.

Devonshire, V., E. Havrdova, et al. (2012). "Relapse and disability outcomes in patients with multiple sclerosis treated with fingolimod: subgroup analyses of the double-blind, randomised, placebo-controlled FREEDOMS study." Lancet neurology **11**(5): 420-428.

Dong, Y. and E. N. Benveniste (2001). "Immune function of astrocytes." Glia **36**(2): 180-190.

Durafourt, B. A., C. Lambert, et al. (2011). "Differential responses of human microglia and blood-derived myeloid cells to FTY720." Journal of neuroimmunology **230**(1-2): 10-16.

Dutta, R. and B. D. Trapp (2011). "Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis." Progress in neurobiology **93**(1): 1-12.

Eugster, H. P., K. Frei, et al. (1998). "IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis." European journal of immunology **28**(7): 2178-2187.

Fischer, I., C. Alliod, et al. (2011). "Sphingosine Kinase 1 and Sphingosine 1-Phosphate Receptor 3 Are Functionally Upregulated on Astrocytes under Pro-Inflammatory Conditions." PloS one **6**(8): e23905.

Foster, C. A., L. M. Howard, et al. (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." The Journal of pharmacology and experimental therapeutics **323**(2): 469-475.

Fukuda, A., S. B. Deshpande, et al. (1998). "Astrocytes are more vulnerable than neurons to cellular Ca2+ overload induced by a mitochondrial toxin, 3-nitropropionic acid." Neuroscience **87**(2): 497-507.

Giussani, P., A. Ferraretto, et al. (2007). "Sphingosine-1-phosphate and calcium signaling in cerebellar astrocytes and differentiated granule cells." Neurochemical research **32**(1): 27-37.

Glabinski, A. R., M. Tani, et al. (1997). "Synchronous synthesis of alpha- and beta-chemokines by cells of diverse lineage in the central nervous system of mice with relapses of chronic experimental autoimmune encephalomyelitis." The American journal of pathology **150**(2): 617-630.

Graler, M. H. and E. J. Goetzl (2004). "The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors." The FASEB journal : official publication of the Federation of American Societies for Experimental Biology **18**(3): 551-553.

Grynkiewicz, G., M. Poenie, et al. (1985). "A new generation of Ca2+ indicators with greatly improved fluorescence properties." The Journal of biological chemistry **260**(6): 3440-3450.

Harris, G. L., M. B. Creason, et al. (2012). "In vitro and in vivo antagonism of a G protein-coupled receptor (S1P3) with a novel blocking monoclonal antibody." PloS one **7**(4): e35129.

Hla, T. and V. Brinkmann (2011). "Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation." Neurology **76**(8 Suppl 3): S3-8.

Holliday, J. and D. L. Gruol (1993). "Cytokine stimulation increases intracellular calcium and alters the response to quisqualate in cultured cortical astrocytes." Brain research **621**(2): 233-241.

John, G. R., S. C. Lee, et al. (2005). "IL-1-regulated responses in astrocytes: relevance to injury and recovery." Glia **49**(2): 161-176.

Kimura, A., T. Ohmori, et al. (2008). "Antagonism of sphingosine 1-phosphate receptor-2 enhances migration of neural progenitor cells toward an area of brain." Stroke; a journal of cerebral circulation **39**(12): 3411-3417.

Kipp, M. and S. Amor (2012). "FTY720 on the way from the base camp to the summit of the mountain: relevance for remyelination." Multiple sclerosis **18**(3): 258-263.

Kulakowska, A., M. Zendzian-Piotrowska, et al. (2010). "Intrathecal increase of sphingosine 1-phosphate at early stage multiple sclerosis." Neuroscience letters **477**(3): 149-152.

Lee, C. W., J. W. Choi, et al. (2010). "Neurological S1P signaling as an emerging mechanism of action of oral FTY720 (fingolimod) in multiple sclerosis." Archives of pharmacal research **33**(10): 1567-1574.

Liberto, C. M., P. J. Albrecht, et al. (2004). "Pro-regenerative properties of cytokine-activated astrocytes." Journal of neurochemistry **89**(5): 1092-1100.

Maceyka, M., K. B. Harikumar, et al. (2012). "Sphingosine-1-phosphate signaling and its role in disease." Trends in cell biology **22**(1): 50-60.

Markowitz, C. E. (2007). "Interferon-beta: mechanism of action and dosing issues." Neurology **68**(24 Suppl 4): S8-11.

May, L. T., K. Leach, et al. (2007). "Allosteric modulation of G protein-coupled receptors." Annual review of pharmacology and toxicology **47**: 1-51.

Meyer Zu Heringdorf, D. (2004). "Lysophospholipid receptor-dependent and -independent calcium signaling." Journal of cellular biochemistry **92**(5): 937-948.

Miron, V. E., A. Schubart, et al. (2008). "Central nervous system-directed effects of FTY720 (fingolimod)." Journal of the neurological sciences **274**(1-2): 13-17.

Mullershausen, F., L. M. Craveiro, et al. (2007). "Phosphorylated FTY720 promotes astrocyte migration through sphingosine-1-phosphate receptors." Journal of neurochemistry **102**(4): 1151-1161.

Mullershausen, F., F. Zecri, et al. (2009). "Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors." Nature chemical biology **5**(6): 428-434.

Nag, S. (2011). "Morphology and properties of astrocytes." Methods in molecular biology **686**: 69-100.

Nair, A., T. J. Frederick, et al. (2008). "Astrocytes in multiple sclerosis: a product of their environment." Cellular and molecular life sciences : CMLS **65**(17): 2702-2720.

Olivera, A., T. Kohama, et al. (1999). "Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival." The Journal of cell biology **147**(3): 545-558.

Oo, M. L., S. Thangada, et al. (2007). "Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor." The Journal of biological chemistry **282**(12): 9082-9089.

Osinde, M., F. Mullershausen, et al. (2007). "Phosphorylated FTY720 stimulates ERK phosphorylation in astrocytes via S1P receptors." Neuropharmacology **52**(5): 1210-1218.

Pebay, A., M. Toutant, et al. (2001). "Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades." The European journal of neuroscience **13**(12): 2067-2076.

Pekny, M. and M. Pekna (2004). "Astrocyte intermediate filaments in CNS pathologies and regeneration." The Journal of pathology **204**(4): 428-437.

Prat, A., K. Biernacki, et al. (2001). "Glial cell influence on the human blood-brain barrier." Glia **36**(2): 145-155.

Pyne, S. and N. J. Pyne (2002). "Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors." Biochimica et biophysica acta **1582**(1-3): 121-131.

Ramgolam, V. S., Y. Sha, et al. (2009). "IFN-beta inhibits human Th17 cell differentiation." Journal of immunology **183**(8): 5418-5427.

Ransohoff, R. M., T. A. Hamilton, et al. (1993). "Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune

encephalomyelitis." FASEB journal : official publication of the Federation of American Societies for Experimental Biology **7**(6): 592-600.

Rapizzi, E., C. Donati, et al. (2007). "Sphingosine 1-phosphate receptors modulate intracellular Ca2+ homeostasis." Biochemical and biophysical research communications **353**(2): 268-274.

Rzigalinski, B. A., S. Liang, et al. (1997). "Effect of Ca2+ on in vitro astrocyte injury." Journal of neurochemistry **68**(1): 289-296.

Selmaj, K. W., M. Farooq, et al. (1990). "Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor." Journal of immunology **144**(1): 129-135.

Sidoryk-Wegrzynowicz, M., M. Wegrzynowicz, et al. (2011). "Role of astrocytes in brain function and disease." Toxicologic pathology **39**(1): 115-123.

Sorensen, S. D., O. Nicole, et al. (2003). "Common signaling pathways link activation of murine PAR-1, LPA, and S1P receptors to proliferation of astrocytes." Molecular pharmacology **64**(5): 1199-1209.

Sorensen, T. L., C. Trebst, et al. (2002). "Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system." Journal of neuroimmunology **127**(1-2): 59-68.

Spooren, A., R. Kooijman, et al. (2010). "Cooperation of NFkappaB and CREB to induce synergistic IL-6 expression in astrocytes." Cellular signalling **22**(5): 871-881.

Suzuki, S., S. Enosawa, et al. (1996). "A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation." Transplantation **61**(2): 200-205.

Van Doorn, R., J. Van Horssen, et al. (2010). "Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions." Glia **58**(12): 1465-1476.

Verzijl, D., S. L. Peters, et al. (2010). "Sphingosine-1-phosphate receptors: zooming in on ligand-induced intracellular trafficking and its functional implications." Molecules and cells **29**(2): 99-104.

Williams, K. C., N. P. Dooley, et al. (1995). "Antigen presentation by human fetal astrocytes with the cooperative effect of microglia or the microglial-derived

cytokine IL-1." The Journal of neuroscience : the official journal of the Society for Neuroscience **15**(3 Pt 1): 1869-1878.

Yoon, C. M., B. S. Hong, et al. (2008). "Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways." Blood **112**(4): 1129-1138.

zu Heringdorf, D. M., M. E. Vincent, et al. (2003). "Inhibition of Ca(2+) signalling by the sphingosine 1-phosphate receptor S1P(1)." Cellular signalling **15**(7): 677-687.