FTY720 ( fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation

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Sphingosine 1-phosphate (S1P), a lysophospholipid, has gained relevance to multiple sclerosis through the discovery of FTY720 ( fingolimod), recently approved as an oral treatment for relapsing forms of multiple sclerosis. Its mechanism of action is thought to be immunological through an active phosphorylated metabolite, FTY720-P, that resembles S1P and alters lymphocyte trafficking through receptor subtype S1P1. However, previously reported expression and in vitro studies of S1P receptors suggested that direct CNS effects of FTY720 might theoretically occur through receptor modulation on neurons and glia. To identify CNS cells functionally contributing to FTY720 activity, genetic approaches were combined with cellular and molecular analyses. These studies relied on the functional assessment, based on clinical score, of conditional null mutants with cellular and molecular analyses. These studies relied on the functional assessment, based on clinical score, of conditional null mutants displaying WT lymphocyte trafficking that responded normally to FTY720. In marked contrast, EAE was attenuated and FTY720 efficacy was lost in mutants lacking S1P1 on GFAP-expressing astrocytes but not on neurons. In situ hybridization studies confirmed that astrocyte loss of S1P1 was the key alteration in functionally affected mutants. Reductions in EAE clinical scores were paralleled by reductions in demyelination, axonal loss, and astrogliosis. Receptor rescue and pharmacological experiments supported the loss of S1P1 on astrocytes through functional antagonism by FTY720-P as a primary FTY720 mechanism. These data identify nonimmunological CNS mechanisms of FTY720 efficacy and implicate S1P signaling pathways within the CNS as targets for multiple sclerosis therapies.

Results

A genetic approach using an engineered, floxed S1P1 allele (Fig. S1) generated conditionally null mutants after crosses with previously established transgenic mice expressing different cell lineage promoters driving Cre recombinase for all CNS lineages (Nestin-Cre) (23), neurons (Synapsin-Cre) (24), or GFAP-expressing cell lineages, particularly in spinal cord white matter astrocytes (GFAP-Cre) (25, 26). All mutants were back-crossed into C57BL/6j to allow assessment of these mutants when (i) challenged by EAE and (ii) therapeutically exposed to FTY720. Characterization of these conditional mutants showed the removal of S1P1 from the targeted lineages (Fig. S1).

Monophasic EAE was produced in mice by immunization with myelin oligodendrocyte glycoprotein (MOG). MOG-challenged WT C57BL/6j (Fig. S1A and B) or recombinant control mice Sipre−/− (Fig. 1 C, E, and G) showed robust EAE clinical signs using conservative scoring criteria compared with S1P1−/− (Fig. 1 D, F, and H), and S1P1 was genetically deleted from specific subsets of CNS cell types questioned by EAE challenge combined with pharmacological, histological, cellular, and biochemical analyses. These studies implicate a primary CNS locus for FTY720 activity through S1P1 in astrocytes.

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mencing 12–15 d after immunization, with clinical signs present through experiment termination (80 d) (Fig. S2B). S1P₁-dependent FTY720 efficacy was examined by assessing its known immunological effects in preventing lymphocyte egress from lymphoid compartments to produce PBL depletion (13, 14). This effect has been proposed as the predominant mechanism of action for FTY720 (11, 16). Consistent with prior reports, FTY720 administration after EAE disease onset reduced both clinical scores and PBL counts (11, 13, 27) (Fig. 1).

Rather, clinical scores were refractory to FTY720 treatment (Fig. 1).

To identify involved CNS cell types, FTY720 efficacy was assessed using conditional null mutants for S1P₁ in neurons (S1pr1loxP;GFAP-Cre; GFAP-Cre; n = 12) compared with littermate controls (n = 7; daily administration of 3 mg/kg FTY720). Reduction of PBLs by FTY720 at 24 h (percentage at 24 h relative to time = 0; 3 mg/kg FTY720 exposure obtained from animals in G (n = 6 for each group)). Mean clinical score of EAE induced in astrocyte S1P₁, conditional null mutants (S1pr1loxP; GFAP-Cre; n = 12) compared with littermate controls (n = 7; daily administration of 3 mg/kg FTY720). (D) Reduction of PBLs by FTY720 at 24 h (percentage at 24 h relative to time = 0; 3 mg/kg FTY720 exposure obtained from animals in G (n = 6 for each group)). Mean clinical score of EAE induced in neuronal S1P₁ null mutants (S1pr1loxP; synapsin-cre; n = 12) compared with littermate controls (n = 7; daily administration of 3 mg/kg FTY720). (E) Mean clinical score of EAE induced in neuronal S1P₁, conditional null mutants (S1pr1loxP; Synapsin-Cre; GFAP-Cre; n = 12) compared with littermate controls (n = 7; daily administration of 3 mg/kg FTY720). (F) Reduction of PBLs by FTY720 at 24 h (percentage at 24 h relative to time = 0; 3 mg/kg FTY720 exposure obtained from animals in G (n = 6 for each group)).
Fig. 2. S1P1 gene expression and Cre-mediated conditional loss as detected by in situ hybridization identify astrocytes in the CNS. In situ hybridization shows S1pr1 expression (dark label) throughout the adult CNS (shown for cerebellum, brainstem, and spinal cord) in WT mice (A, E, and I) that is lost after conditional deletion in S1pr1loxP/loxP, Nestin-Cre (B, F, and J) and S1pr1loxPloxP, GFAP-Cre (C, G, and K) but not in S1pr1loxPloxP, Synapsin-Cre (D, H, and L) mice. In WT mice, S1pr1 message is abundant in the Bergmann glia of the Purkinje layer in the cerebellum (A), which was confirmed by double labeling with a GFAP antibody (A Inset; red; P, Purkinje neuron) and not observed in other neuronal populations (G, granule neurons). In brainstem (E–H) and the white matter of spinal cord (I–L), S1pr1 message is present (arrows) in WT (l) and S1pr1loxPloxP, Synapsin-Cre (l) but is not detected when conditionally deleted in Nestin-Cre (J) or GFAP-Cre (K) cells. Labeling is occasionally observed in presumptive endothelial cells (arrowheads). (M–O) Double-labeling cells using S1pr1 in situ hybridization, combined with GFAP immunolabeling, confirms S1pr1 expression in spinal cord astrocytes. In situ hybridization (M, dark label), GFAP immunolabeling (N, red), and merged image (O). (Scale bars: A–D, 100 μm; E–L, 50 μm; M–O, 20 μm.)

Purkinje neurons of the cerebellum as a major brain locus for normal S1P1 gene expression (28, 29), and this general pattern was reproduced (Fig. 2A). However, assessment of each mutant genotype compared with controls (Fig. 2A–D) produced a surprising result, where neuronal deletion (by Synapsin-Cre) had no effect on cerebellar labeling, labeling was eliminated by pan-neuronal and glial (Nestin-Cre) as well as astrocyte-enriched deletion (GFAP-Cre). Higher magnification views of the cerebellar labeling revealed that Purkinje neurons were not labeled, whereas labeling occurred in surrounding cells that were consistent with being Bergmann glia, which are a population of cerebellar astrocytes (31), an identity confirmed by double-labeling cells with GFAP (immunolabeling) and S1P1 (in situ hybridization) (Fig. 2A Inset). Consistent with cerebellar astrocyte S1P1 gene expression, in situ hybridization for S1P1 was mostly eliminated throughout the neuraxis [brainstem (Fig. 2E–H) and spinal cord (Fig. 2I–L)] after conditional deletion by Nestin- or GFAP-Cre but remained at control levels after neuronal deletion using Synapsin-Cre. Astrocyte identification was supported by double labeling for S1P1 and GFAP (Fig. 2A Inset and M–O). No obvious neuronal labeling was observed. Overall, these data effectively eliminate neuronal contributions of S1P1 signaling to account for the observed functional alterations in EAE clinical scores of Nestin- or GFAP-Cre conditional mutants, and they identify GFAP-expressing astrocytes as both the major cell type expressing S1P1 in the adult CNS and that which is affected by S1P1 signaling produced during FTY720 exposure.

This astrocyte identification is consistent with astroglisis, identified by GFAP immunolabeling (32, 33), which occurs during both EAE (34) and multiple sclerosis (32, 35–37). Notably, astroglisis can precede clinical manifestations and immune cell infiltration in EAE (38). Therefore, histological analyses on clinically defined CNS regions of the lumbar spinal cord were pursued. In normal EAE controls (Fig. 3 and Fig. S3), prominent astroglisis was identified by increased GFAP immunoreactivity (Fig. 3B, GFAP and Fig. S3D) along with increased spinal cord cell density, particularly in white matter tracts (Fig. 3B, DAPI and Fig. S3 B and C), as well as increased numbers of CD11b-positive cells (microglia or macrophages) (Fig. S3E) and lymphocytes (Fig. S3F) within lesion sites. Remarkably, FTY720 exposure before (Fig. 3E) or during (Fig. 3F and Fig. S3H) EAE prevented or reduced astroglisis accompanying increases in cell density as did specific deletion of S1P1 by Nestin- or GFAP-Cre (Fig. 3D and Figs. S3 and S4A). Accumulation of immune cells was also markedly reduced by genetic S1P1 deletion (Fig. S3G) or FTY720 exposure (Fig. S3H). These data indicate that astroglisis and some aspects of the immune response are promoted through astrocyte S1P1 signaling and are reduced by genetic removal of S1P1 from astrocytes as well as through FTY720 exposure.

Astroglisis has also been reported after direct S1P injection into the CNS (39) or after spinal cord injury associated with increased S1P levels (40). S1P levels were assessed in WT or Nestin-Cre S1P1-conditional EAE spinal cords by HPLC/MS analysis. S1P content in EAE spinal cords was approximately twofold higher in WT EAE vs. WT naïve (non-EAE) mice (Fig. 3G), consistent with an astrogliosis–S1P link. Notably, S1P levels were reduced in Nestin-Cre S1P1 conditional null mutants (Fig. 3G), suggesting links between S1P levels and astrocyte S1P1 signaling.

Proinflammatory cytokines are a major factor in EAE and MS, particularly IL-1β, IL-6, and IL-17 (32, 41). Expression levels for these cytokines were examined by assessing both protein (Fig. 3H) and mRNA (Fig. S3 I–K) levels. All were increased in WT mice during EAE (Fig. 3H and Fig. S3 I–K) but reduced by Nestin-Cre deletion of S1P1, and by FTY720 administration (Fig. 3H and Fig. S3 I–K). These data indicate that FTY720 exposure can regulate proinflammatory cytokine production during EAE through astrocyte S1P1 signaling.

Demyelination and neurodegeneration are hallmarks of EAE and multiple sclerosis (1). Spinal cords from control and S1P1 conditional null mutant EAE produced by Nestin- or GFAP-Cre (Fig. 3 and Fig. S4) were assessed by fluoromyelin staining and neurofilament immunolabeling; both mutations produced similar results. Levels of EAE-induced demyelination (Fig. 3I) and ax-
S1pr1loxP/loxP, Nestin-Cre

GFAP

Naive

EAE

EAE+FTY720

Demyelination was reduced by S1P1 deletion or FTY720 exposure as measured by HPLC/MS. Naive (WT; n = 6), EAE (WT; n = 7; mean clinical score at examination = 2), and EAE (S1pr1loxP/loxP; nestin-cre; n = 5; mean clinical score at examination = 0.6). *P < 0.006 and **P < 0.0004 (t-test). (H) Increased cytokine protein levels (IL-1β, IL-6, and IL-17) in the lumbar spinal cord after EAE challenge were reduced in S1P1 conditional null mutants (S1pr1loxP/loxP; nestin-cre) or after FTY720 exposure as measured by ELISA. Naive (WT; n = 8), EAE (WT; n = 7; mean clinical score at examination = 2), EAE (S1pr1loxP/loxP; nestin-cre; n = 5; mean clinical score at examination = 0.5), and EAE+FTY720 (WT; n = 8; mean clinical score at examination = 0.4). *P < 0.04, **P < 0.001 (vs. naive), #P < 0.05, ##P < 0.006, and ###P < 0.001 (vs. EAE; t-test) (Fig. S3 I–K). (I) Demyelination was reduced by S1P1 deletion (S1pr1loxP/loxP; nestin-cre) or FTY720 exposure as identified by fluoromyelin staining. (J) Axonal damage was reduced by S1P1 deletion or FTY720 exposure as identified by antineurofilament immunolabeling. Use of an independent Cre driver that removed S1P1 from astrocytes (S1pr1loxP/loxP; GFAP-cre) produced similar results (Fig. S4). (Scale bar: 100 μm.)

Fig. 3. Reduction of pathological and biochemical sequelae of EAE by S1P1 deletion produced by Nestin-Cre or FTY720 exposure. GFAP immunoreactivity identified by anti-GFAP immunolabeling (green) in the ventral lumbar spinal cord compared with DAPI staining (blue for all panels). (A) Control (S1pr1loxP/loxP) naive (non-EAE). (B) Control (S1pr1loxP/loxP) EAE (clinical score at examination = 1.5). Arrowheads and arrows indicate areas of GFAP immunoreactivity and increased cell density, respectively. (C) S1P1 conditional null mutant (S1pr1loxP/loxP; nestin-cre) naive. (D) S1P1 conditional null mutant (S1pr1loxP/loxP; nestin-cre) EAE-induced (clinical score at examination = 0.5). (E) Control pretreated with FTY720 followed by EAE challenge (clinical score at examination = 0.5). (F) Control challenged with EAE followed by FTY720 (clinical score at examination = 1.5 before and = 0.5 after FTY720 administration) (Fig. S3 A–H). (G) S1P levels in the lumbar spinal cord were increased after EAE challenge but reduced in S1P1 conditional null mutants (S1pr1loxP/loxP; nestin-cre) as measured by HPLC/MS. Naive (WT; n = 6), EAE (WT; n = 7; mean clinical score at examination = 2), and EAE (S1pr1loxP/loxP; nestin-cre; n = 5; mean clinical score at examination = 0.6). *P < 0.006, **P < 0.0004 (t-test). (H) Increased cytokine protein levels (IL-1β, IL-6, and IL-17) in the lumbar spinal cord after EAE challenge were reduced in S1P1 conditional null mutants (S1pr1loxP/loxP; nestin-cre) or after FTY720 exposure as measured by ELISA. Naive (WT; n = 8), EAE (WT; n = 7; mean clinical score at examination = 2), EAE (S1pr1loxP/loxP; nestin-cre; n = 5; mean clinical score at examination = 0.5), and EAE+FTY720 (WT; n = 8; mean clinical score at examination = 0.4). *P < 0.04, **P < 0.001 (vs. naive), #P < 0.05, ##P < 0.006, and ###P < 0.001 (vs. EAE; t-test) (Fig. S3 I–K). (I) Demyelination was reduced by S1P1 deletion (S1pr1loxP/loxP; nestin-cre) or FTY720 exposure as identified by fluoromyelin staining. (J) Axonal damage was reduced by S1P1 deletion or FTY720 exposure as identified by antineurofilament immunolabeling. Use of an independent Cre driver that removed S1P1 from astrocytes (S1pr1loxP/loxP; GFAP-cre) produced similar results (Fig. S4). (Scale bar: 100 μm.)
sine kinase 2 (Sphk2) (6), which is expressed in these astrocytes (Fig. S6B), consistent with S1P1 effects activated by exogenously applied FTY720 or FTY720-P. As with FTY720, exposure to either FTY720-P or AU954 also produced prolonged internalization of S1P1 (Fig. 4A, 2 h). These data were recapitulated in the astrocyte-like glioma cell line, C6, using S1P1 overexpression (Fig. 4B) that allowed prolonged examination during compound exposure compared with use of primary astrocytes. The sustained receptor internalization, consistent with functional antagonism, was maintained in the presence of FTY720, FTY720-P, and AU954, similar to the results obtained in astrocytes.

**Discussion**

The aggregate results support functional antagonism of astrocyte S1P1 rather than forms of agonism as the predominant receptor mechanism for FTY720 efficacy. Receptor internalization of FTY720-P-S1P1 associated with a report of persistent agonism (10) is not likely to be relevant, because genetic as well as pharmacological loss of CNS S1P1 signaling both reduced EAE clinical scores and histological sequelae, including astrogliosis, contrasting with S1P receptor agonism in the CNS that promotes astrogliosis (39, 40, 44). Persistent S1P1 signaling may be relevant to the actions of FTY720 on lymphocyte egress (10); however, the separation of immunological and CNS S1P1 activities observed here supports functional antagonism within the CNS as the primary mechanism of action. It is notable that these distinguishable CNS effects may have particular relevance for primary progressive forms of multiple sclerosis that are refractory to immunomodulatory therapies (45).

In the adult CNS, in situ hybridization and conditional deletion data support GFAP-expressing astrocytes as the major cell type influencing EAE-associated behavioral, histological, and biochemical endpoints. Reduced astrogliosis associated with S1P1 loss along with reduced cytokine expression and S1P levels identify possible downstream mechanisms activated by S1P1 loss or FTY720 exposure. Other nonmutually exclusive mechanisms downstream of S1P signaling in astrocytes include astrocyte effects on the blood–brain barrier or possible neuroprotective actions, all of which deserve further investigation. S1P1 has also been reported to be expressed by oligodendrocytes (46), and under some conditions, neurons (19, 28, 29). This deserves closer analysis in view of the clarified expression of S1P1 on Bergmann glia rather than Purkinje neurons identified here, as well as non-CNS cell lineages that include microglia (47) and endothelial cells (9), all of which may have roles at discrete phases of EAE or multiple sclerosis. The role of other expressed S1P receptor subtypes remains to be assessed.

In summary, this study identifies a nonimmunological CNS mechanism of action for FTY720 in EAE and likely, multiple sclerosis: loss of S1P1 signaling from astrocytes (Fig. S7). Genetic, in situ hybridization, and receptor internalization data identify astrocytes as the primary CNS cell type affected by S1P1 modulation that is relevant to FTY720 efficacy. These effects seem to be largely distinct from the previously known effects of FTY720 on lymphocyte trafficking, results that may in part explain paradoxical human clinical data that showed increasing PBL depletion with increasing FTY720 dose (48) but unchanged or increasing efficacy of multiple sclerosis with decreasing FTY720 dose. The CNS activities observed here may also contribute to the as yet unexplained reductions in brain atrophy observed in human clinical trials (2, 3) that could hypothetically reflect CNS tissue preservation and neuroprotection. Thus, modulation of S1P receptor-influenced pathways within the CNS may offer strategies that could reduce or avoid immunosuppressive side effects common to current treatments and potentially provide neuroprotection towards the development of improved multiple sclerosis therapies.

**Materials and Methods**

**EAE Induction and Drug Treatment.** All animal protocols were approved by the Animal Research Committee (IACUC) of The Scripps Research Institute (TSRI) and conformed to National Institutes of Health guidelines and public law. EAE was induced by MOG amino acid 35–55 (MEGVYRSPFSRVVHLYRNGK, 98% purity; American Peptide) immunization in 7- to 9-wk-old female mice: WT mice (C57BL/6) and conditional null mutants for S1P1, in different CNS cell lineages, including S1pr1loxP/loxP; nestin-cre (S1P1 deletion in all CNS cell lineages) (23), S1pr1loxP/loxP; synapsin-cre (S1P1 deletion in neuronal lineages) (24), and S1pr1loxP/loxP; GFAP-cre (S1P1 deletion in astrocyte lineages) (25, 26) or littermate control mice (S1pr1loxP/loxP). For immunization, MOG was dissolved in water, and an emulsion was prepared in Complete Freund Adjuvant (CFA). The emulsion of MOG (200 μg) in CFA was injected s.c., with an i.p. injection of Bordetella pertussis toxin (PTX; 400 ng PTX were injected into mice at days 0 and 2 after MOG/CFA injection), and then, mice were weighed and monitored daily.

**PBL Counts.** Mice were anesthetized with isoflurane before blood collection by tail tip transection. Whole-blood samples (200–300 μl) were collected into EDTA blood collection tubes (BD Biosciences) and analyzed in duplicate on a Hemavet 850FS Multi Species Hematology System (Drew Scientific) programmed with mouse hematology settings.

**Histological Analysis.** Tissues were prepared by rapid dissection, embedded in Tissue-Tek Optimal Cutting Temperature (OCT) (Ted Pella, Inc., Redding, CA) compound, and rapidly frozen on powdered dry ice. Cryostat sections (20 μm) were fixed, and native fluorescence was detected. C6 glioma cells allowed extended examination during compound exposure compared with use of primary astrocytes. The sustained receptor internalization in both primary astrocytes and C6 cells is consistent with S1P1 functional antagonism in the presence of FTY720, FTY720-P, and AU954, contrasting with receptor recycling observed with S1P. Asterisks identify cytosolic locations, whereas arrows indicate the cell surface. (Scale bars: A, 10 μm; B, 20 μm.)
µm were fixed (10 min) in 4% paraformaldehyde (PFA), permeabilized in 0.5% Triton X-100, and blocked with 3% BSA. Tissue sections were labeled with primary antibody directed against anti-GFP (11,000; Sigma) and rabbit antineurofilament 200 (1:200; Serotec) were used for detecting astrogliosis and axonal damage, respectively. To detect demyelination, tissue sections were stained with fluoroMyelin (1:300; Invitrogen). Sections were visualized directly or incubated with FITC-conjugated anti-igG (1:2,000; Molecular Probes) and counterstained with DAPI (5 μg/ml). In situ hybridization for S1pr1 in the cerebellum, spinal cord, and brainstem of unmanipulated and various S1pr1 conditional null mutants used tissues sectioned at 20 μm and hybridized with an S1pr1-specific digoxigenin (DIG)-labeled antisense probe followed by colorimetric detection. Double labeling of tissue sections for immunohistochemistry used an anti-GFP antibody and subsequent FITC-conjugated second antibody. Images were collected with an AxioCam digital camera (Zeiss) and prepared using Adobe Photoshop version 8.0.

Measurements of S1P in Spinal Cord. Lumbar spinal cord was homogenized with ice-cold methanol (MeOH), centrifuged, and used for S1P extraction in the presence of 1 μM C17 S1P (Avanti Polar Lipids) as an internal standard. Samples were processed at the TSRI Center for Mass Spectrometry using an Agilent 6410 triple quad mass spectrometer coupled to an Agilent 1100 LC/MS system. S1P levels were calculated with the internal standard, and the values were represented as picomoles per milligram of tissue.

Determination of Cytokine Levels by ELISA. Lumbar spinal cord protein samples were used to determine cytokine levels using ELISA kits: IL-1β (BD Pharmingen), IL-6 (Millipore), and IL-17 (R&D System). Protein levels of IL-1α and IL-17 were determined by absorbance at 450 nm and calculated based on appropriate standard curves. Protein levels of IL-6 were obtained from Millipore using Luminox.

Determination of S1P, Internalization and Recycling in Astrocytes. Primary astrocytes were isolated from cerebral cortices of S1P1 conditional KO mice and retrovirally transduced with an S1P–EGFP fusion construct. Cells were labeled with mouse anti-GFP and rabbit anti-GFP and visualized with fluorescence-conjugated anti-igG. S1P localization in cells was determined by confocal microscopy equipped with the Fluoview program (Leica). The astrocyte cell line (C6 glioma cells) was similarly processed.

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