FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 ($S1P_1$) 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 $S1P_1$. 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 mouse mutants lacking $S1P_1$ in CNS cell lineages and challenged by experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. All conditional null mutants displayed WT lymphocyte trafficking that responded normally to FTY720. In marked contrast, EAE was attenuated and FTY720 efficacy was lost in CNS mutants lacking $S1P_1$ on GFAP-expressing astrocytes but not on neurons. In situ hybridization studies confirmed that astrocyte loss of $S1P_1$ 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 $S1P_1$ 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.

G protein-coupled receptor | knockout | lysophospholipid | neuroprotection

Multiple sclerosis is an autoimmune disorder characterized by CNS demyelination, inflammation, and neurodegeneration (1). Current Food and Drug Administration (FDA)-approved therapies predominantly target immunological pathways through injectable agents. FTY720 (known clinically as fingolimod) is an oral drug that has shown efficacy in human multiple sclerosis clinical trials (2, 3), and in September 2010, received FDA approval as an oral therapy for relapsing forms of multiple sclerosis. The actions of FTY720 seem to involve at least two, paradoxically, opposite sphingosine 1-phosphate (S1P) receptor mechanisms operating in the immune system: agonism and functional antagonism. FTY720 is phosphorylated in vivo to produce the active metabolite FTY720-P, which is a nonselective S1P receptor agonist for four of the five known S1P receptors (4–6), and can function as an agonist in vivo (7). However, FTY720-P can also act as a functional antagonist, where bound S1P receptors are irreversibly internalized and degraded rather than recycled back to the cell surface as occurs with S1P ligands (8, 9); additionally, FTY720-P signaling may persist even after receptor internalization (10). The biological locus responsible for FTY720 efficacy is believed to be immunological, whereby FTY720-P, acting through the receptor subtype known as $S1P_1$, prevents lymphocyte egress from lymphoid organs, thus inhibiting CNS attack by pathogenic lymphocytes (4, 11–17).

In addition to immunological mechanisms, data also exist for nonimmunological FTY720 mechanisms involving the CNS in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis. These include S1P receptor gene expression in the CNS (18, 19), a discordance between peripheral blood lymphocyte (PBL) levels and FTY720 clinical efficacy in EAE-induced mice (12), drug enrichment in the brain (19), effects on cultured CNS cells (20), sphingolipid CNS metabolic defects in some multiple sclerosis patients (21), and expression of S1P receptors in multiple sclerosis brain lesions (22). CNS mechanisms of FTY720, distinct from immune mechanisms, might represent a way to avoid liabilities of immunosuppression as well as potentially provide neuroprotection. However, no functional in vivo data currently exist to support an immunologically separate CNS mechanism for FTY720, and the identity of functionally involved cell types remains unknown. To address this issue, $S1P_1$ was genetically deleted from specific subsets of CNS cell types followed by EAE challenge combined with pharmacological, histological, cellular, and biochemical analyses. These studies implicate a primary CNS locus for FTY720 activity through $S1P_1$ in astrocytes.

Results

A genetic approach using an engineered, floxed $S1P_1$ 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 $S1P_1$ 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. 1*A* and Fig. S2 *A* and *B*) or unrecombined control mice ($S1pr1^{loxP/loxP}$) (Fig. 1 *C*, *E*, and *G*) showed robust EAE clinical signs using conservative scoring criteria com-

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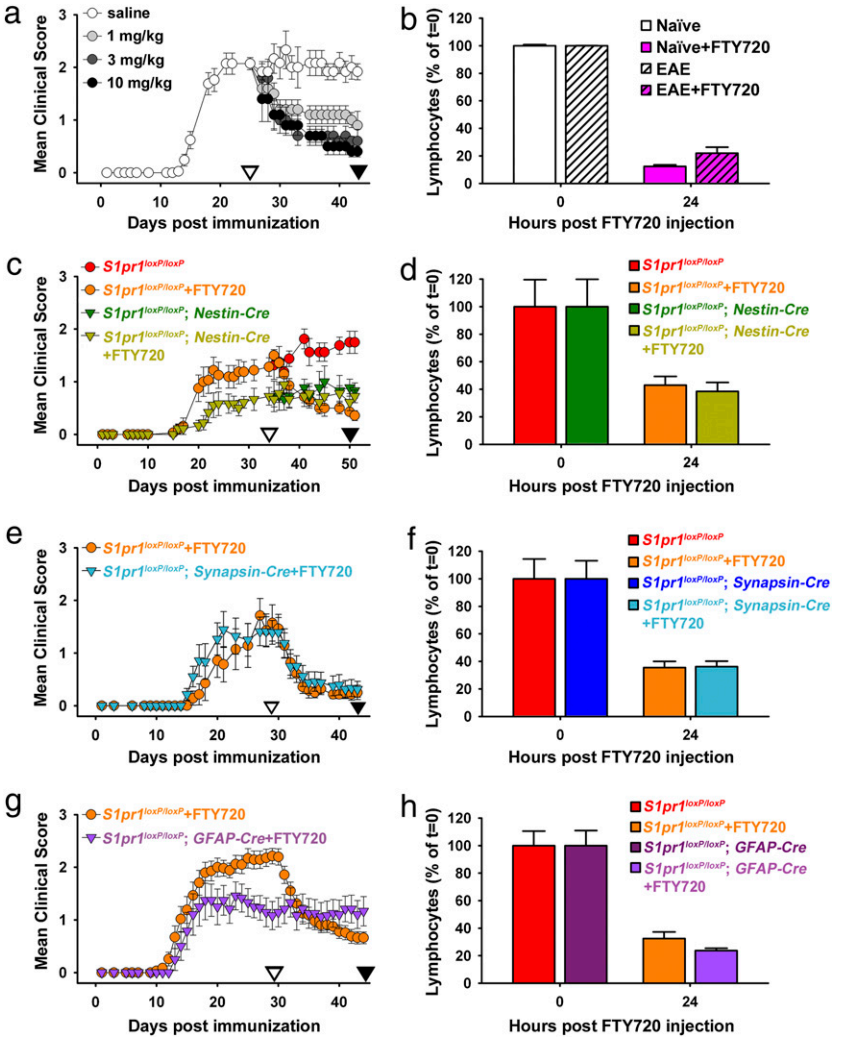

Fig. 1. $S1P_1$ in CNS cell lineages is critical for FTY720 efficacy. (*A*) Mean clinical score of WT EAE mice challenged with FTY720 daily treatment (1, 3, and 10 mg/kg; $n = 5$ for each dose of FTY720) compared with saline control ($n = 6$). (*B*) Reduction of PBLs by FTY720 at 24 h [percentage at 24 h relative to time = 0; 3 mg/kg FTY720 exposure obtained from animals in *A* ($n = 4$ of each group)]. (*C*) Mean clinical score of EAE induced in CNS $S1P_1$ conditional null mutants [$S1pr1^{loxP/loxP}$; *Nestin-Cre*; with FTY720 ($n = 9$) and without FTY720 ($n = 9$)] compared with littermate controls [$S1pr1^{loxP/loxP}$; with FTY720 ($n = 7$) and without FTY720 ($n = 8$); 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 *C* ($n = 3$ for each group)]. (*E*) Mean clinical score of EAE induced in neuronal $S1P_1$ conditional null mutants ($S1pr1^{loxP/loxP}$; *synapsin-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 *E* ($n = 6$ for each group)]. (*G*) Mean clinical score of EAE induced in astrocyte $S1P_1$ conditional null mutants ($S1pr1^{loxP/loxP}$; *GFAP-Cre*; $n = 12$) compared with littermate controls ($n = 27$; daily administration of 3 mg/kg FTY720). (*H*) 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* ($n = 4$ or $n = 3$ for control or conditional mutant mice)]. Data in all figures are represented as mean ± SEM. ▽ and ▼ indicate the start and stop of FTY720 administration, respectively. Actual values for peripheral blood lymphocytes for *B*, *D*, *F*, and *H* were documented (Table S1). Clinical scores of *A*, *C*, *E*, and *G* were statistically analyzed using Mann–Whitney's test (Tables S2–S5).

mencing 12–15 d after immunization, with clinical signs present through experiment termination (80 d) (Fig. S2*B*). $S1P_1$-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 *A* and *B* and Table S1), with both therapeutic and prophylactic efficacy (Fig. S2*C*).

$S1P_1$ expression has been reported on CNS neurons and glia (19, 28, 29), the latter of which includes astrocytes (30). To examine a possible CNS contribution to FTY720 efficacy through $S1P_1$, EAE was induced in conditional null mutant mice where $S1P_1$ was eliminated from both neurons and glia ($S1pr1^{loxP/loxP}$; *Nestin-Cre*) compared with unrecombined littermate controls ($S1pr1^{loxP/loxP}$) followed by FTY720 administration (Fig. 1 *C* and *D* and Table S1). All mice responded to EAE induction, with $S1P_1$ conditional mutants exhibiting a reduced level of disease severity (Fig. 1*C*). PBL depletion after FTY720 administration was indistinguishable in controls vs. mutant mice (Fig. 1*D* and Table S1). If lymphocyte depletion was solely responsible for FTY720 efficacy, then EAE in $S1P_1$ null mutants should have been further reduced; however, this was not observed (Fig. 1*C*). Rather, clinical scores were refractory to FTY720 treatment (Fig. 1*C*), despite the maintained immunological effects on PBL depletion (Fig. 1*D* and Table S1). Moreover, lymph node (LN) cells from $S1pr1^{loxP/loxP}$; *Nestin-Cre* conditional mutants showed normal function as assessed by adoptive transfer experiments, where primed LN cells from EAE-induced unrecombined controls ($S1pr1^{loxP/loxP}$) and conditional null mice induced host disease (Fig. S2*D*). These data indicate that immunological mechanisms are insufficient to fully account for the efficacy of FTY720 and support contributions by CNS $S1P_1$ signaling.

To identify involved CNS cell types, FTY720 efficacy was assessed using conditional null mutants for $S1P_1$ in neurons ($S1pr1^{loxP/loxP}$; *Synapsin-Cre*) (Fig. S1*J*) compared with astrocytes ($S1pr1^{loxP/loxP}$; *GFAP-Cre*) (Fig. S1*K*) challenged by EAE. Neuronal $S1P_1$ null mutants responded like littermate controls (Fig. 1*E*) and displayed normal lymphocyte depletion (Fig. 1*F* and Table S1). In marked contrast, mice with conditional deletion of $S1P_1$ in GFAP-expressing cells resulted in a loss of response to FTY720 exposure (Fig. 1*G*), despite normal PBL depletion (Fig. 1*H* and Table S1). Conditional mutants that were not treated with FTY720 exhibited the predicted pattern of stable disease progression, although clinical signs were attenuated when $S1P_1$ was deleted from astrocytes (Fig. S2*E*). These data identified a non-neuronal and likely astrocyte locus for $S1P_1$ signaling.

The Cre mice were chosen based on previously reported gene expression of $S1P_1$ in neurons and glia (19, 28, 29) and the proven specificity of the Cre-driving promoters used for removing genes from these cell types (23–26). To ascertain that $S1P_1$ removal was indeed occurring from embryonic precursor cells (Fig. S1) and targeted adult cell populations, in situ hybridization analyses of control and conditional mutant brains and spinal cords were pursued (Fig. 2). Prior reports had identified

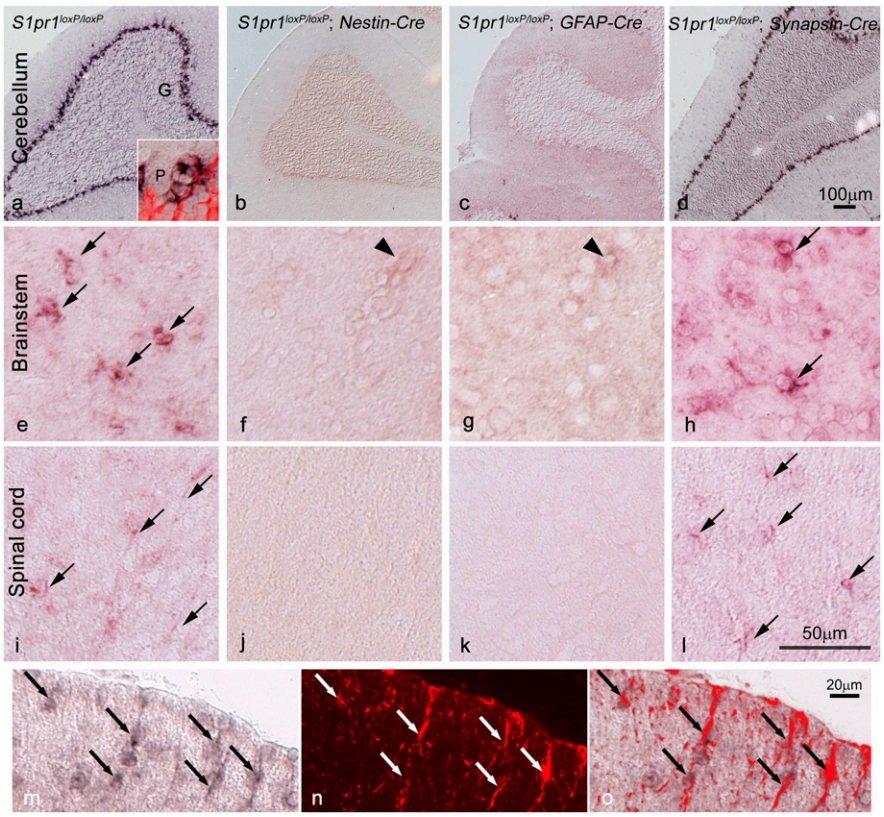

Fig. 2. $S1P_1$ gene expression and Cre-mediated conditional loss as detected by in situ hybridization identify astrocytes in the CNS. In situ hybridization shows *S1pr1* expression (dark label) throughout the adult CNS (shown for cerebellum, brainstem, and spinal cord) in WT mice (*A*, *E*, and *I*) that is lost after conditional deletion in *S1pr1*$^{loxP/loxP}$; *Nestin-Cre* (*B*, *F*, and *J*) and *S1pr1*$^{loxP/loxP}$; *GFAP-Cre* (*C*, *G*, and *K*) mice but not in *S1pr1*$^{loxP/loxP}$; *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 (*I*) and *S1pr1*$^{loxP/loxP}$; *Synapsin-Cre* (*L*) mice 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 $S1P_1$ gene expression (28, 29), and this general pattern was reproduced (Fig. 2*A*). However, assessment of each mutant genotype compared with controls (Fig. 2 *A–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 $S1P_1$ (in situ hybridization) (Fig. 2*A Inset*). Consistent with cerebellar astrocyte $S1P_1$ gene expression, in situ hybridization for $S1P_1$ was mostly eliminated throughout the neuraxis [brainstem (Fig. 2 *E–H*) and spinal cord (Fig. 2 *I–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 $S1P_1$ and GFAP (Fig. 2*A Inset* and *M–O*). No obvious neuronal labeling was observed. Overall, these data effectively eliminate neuronal contributions of $S1P_1$ 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 $S1P_1$ in the adult CNS and that which is affected by $S1P_1$ signaling produced during FTY720 exposure.

This astrocyte identification is consistent with astrogliosis, identified by GFAP immunolabeling (32, 33), which occurs during both EAE (34) and multiple sclerosis (32, 35–37). Notably, astrogliosis 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 astrogliosis was identified by increased GFAP immunoreactivity (Fig. 3*B*, GFAP and Fig. S3*D*) along with increased spinal cord cell density, particularly in white matter tracts (Fig. 3*B*, DAPI and Fig. S3 *B* and *C*), as well as increased numbers of CD11b-positive cells (microglia or macrophages) (Fig. S3*E*) and lymphocytes (Fig. S3*F*) within lesion sites. Remarkably, FTY720 exposure before (Fig. 3*E*) or during (Fig. 3*F* and Fig. S3*H*) EAE prevented or reduced astrogliosis accompanying increases in cell density as did specific deletion of $S1P_1$ by *Nestin-* or *GFAP-Cre* (Fig. 3*D* and Figs. S3 and S4*A*). Accumulation of immune cells was also markedly reduced by genetic $S1P_1$ deletion (Fig. S3*G*) or FTY720 exposure (Fig. S3*H*). These data indicate that astrogliosis and some aspects of the immune response are promoted through astrocyte $S1P_1$ signaling and are reduced by genetic removal of $S1P_1$ from astrocytes as well as through FTY720 exposure.

Astrogliosis 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* $S1P_1$-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. 3*G*), consistent with an astrogliosis–S1P link. Notably, S1P levels were reduced in *Nestin-Cre* $S1P_1$ conditional null mutants (Fig. 3*G*), suggesting links between S1P levels and astrocyte $S1P_1$ 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. 3*H*) and mRNA (Fig. S3 *I–K*) levels. All were increased in WT mice during EAE (Fig. 3*H* and Fig. S3 *I–K*) but reduced by *Nestin-Cre* deletion of $S1P_1$ and by FTY720 administration (Fig. 3*H* and Fig. S3 *I–K*). These data indicate that FTY720 exposure can regulate proinflammatory cytokine production during EAE through atrocyte $S1P_1$ signaling.

Demyelination and neurodegeneration are hallmarks of EAE and multiple sclerosis (1). Spinal cords from control and $S1P_1$ 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. 3*I*) and ax-

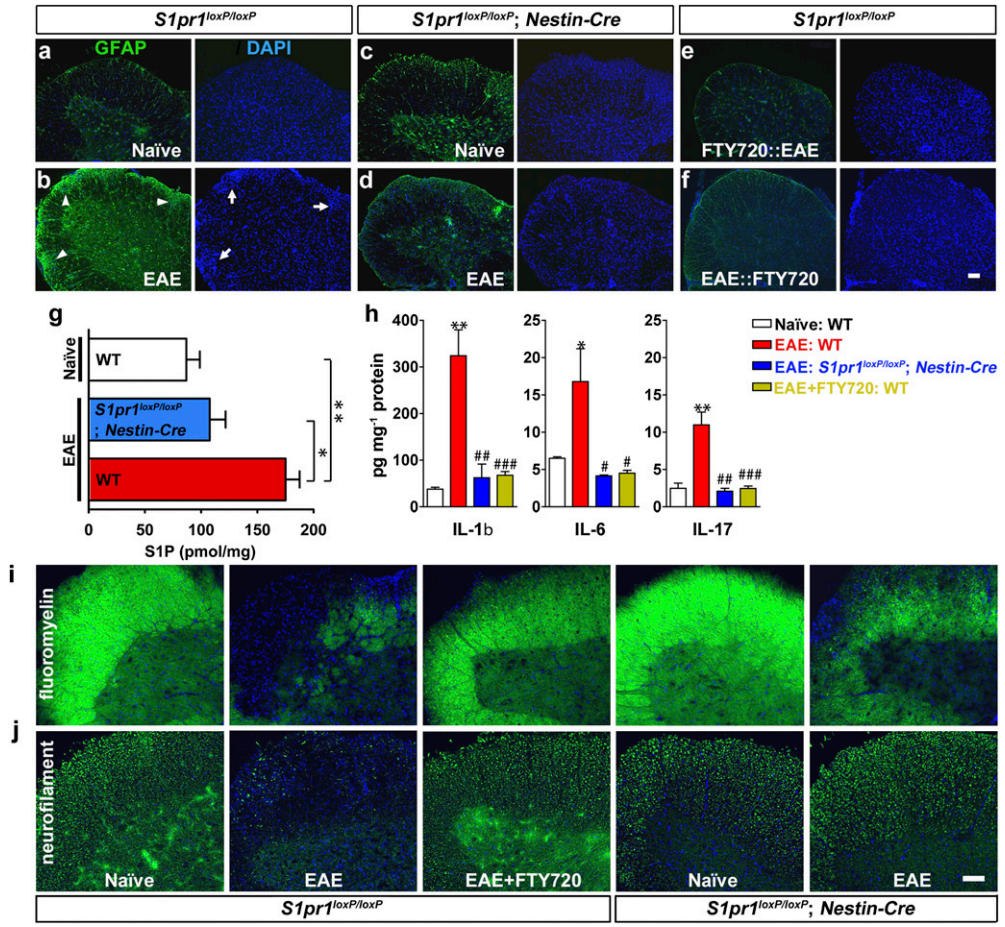

Fig. 3. Reduction of pathological and biochemical sequelae of EAE by $S1P_1$ deletion produced by *Nestin-Cre* or FTY720 exposure. GFAP immunoreactivity identified by anti-GFAP immunolabeling (green) in the ventral lumbar spinal cord compared with DAPI staining (blue for all panels). (*A*) Control (*S1pr1*$^{loxP/loxP}$) naïve (non-EAE). (*B*) Control (*S1pr1*$^{loxP/loxP}$) EAE (clinical score at examination = 1.5). Arrowheads and arrows indicate areas of GFAP immunoreactivity and increased cell density, respectively. (*C*) $S1P_1$ conditional null mutant (*S1pr1*$^{loxP/loxP}$; *nestin-cre*) naïve. (*D*) $S1P_1$ conditional null mutant (*S1pr1*$^{loxP/loxP}$; *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 $S1P_1$ conditional null mutants (*S1pr1*$^{loxP//loxP}$; *nestin-cre*) as measured by HPLC/MS. Naïve (WT; $n = 6$), EAE (WT; $n = 7$; mean clinical score at examination = 2), and EAE (*S1pr1*$^{loxP/loxP}$; *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 $S1P_1$ conditional null mutants (*S1pr1*$^{loxP/loxP}$; *nestin-cre*) or after FTY720 exposure as measured by ELISA. Naïve (WT; $n = 8$), EAE (WT; $n = 7$; mean clinical score at examination = 2), EAE (*S1pr1*$^{loxP/loxP}$; *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. naïve), $^{\#}P < 0.05$, $^{\#\#}P < 0.006$, and $^{\#\#\#}P < 0.001$ (vs. EAE; *t* test) (Fig. S3 *I–K*). (*I*) Demyelination was reduced by $S1P_1$ deletion (*S1pr1*$^{loxP/loxP}$; *nestin-cre*) or FTY720 exposure as identified by fluoromyelin staining. (*J*) Axonal damage was reduced by $S1P_1$ deletion or FTY720 exposure as identified by antineurofilament immunolabeling. Use of an independent Cre driver that removed $S1P_1$ from astrocytes (*S1pr1*$^{loxP/loxP}$; *GFAP-cre*) produced similar results (Fig. S4). (Scale bar: 100 μm.)

onal damage (Fig. 3*J*) were reduced in controls after FTY720 treatment (Fig. 3 *I* and *J*). Similarly, demyelination and axonal damage were also reduced in $S1P_1$-deficient mice produced by *Nestin-Cre* or *GFAP-Cre* (Fig. 3 *I* and *J* and Fig. S4 *B* and *C*). These histological data support protective effects of $S1P_1$ loss from astrocytes or FTY720 exposure.

To address $S1P_1$ function in EAE without potential complications of developmental perturbation produced by genetics, a pharmacological strategy was used with an $S1P_1$-specific agonist, AUY954 (42), in WT EAE. Like FTY720, AUY954 administration during EAE also reduced the clinical severity of EAE (Fig. S5*A*) as well as astrogliosis (Fig. S5*B*). These data independently support a role for $S1P_1$ that complements results obtained from both genetics and FTY720 administration.

The studies presented here identify $S1P_1$ signaling in astrocytes as a key mediator of FTY720 efficacy but also present a paradox: loss of $S1P_1$ signaling by $S1P_1$ deletion ameliorates EAE but so does exposure to S1P receptor agonists FTY720 (i.e., FTY720-P) (5) and AUY954 (42). A single mechanism that accounts for these disparate activities has been reported for FTY720-P in the immune system, where it acts as a functional antagonist, doing so by removing receptors from the cell surface through irreversible internalization after receptor activation rather than the usual receptor recycling back to the cell surface that occurs with S1P itself (8, 9, 13, 43). To assess this mechanism in astrocytes, primary astrocyte cultures were generated from $S1P_1$-deficient mice (Fig. S6*A*) and reconstituted by infection with an EGFP-tagged $S1P_1$ retrovirus. Brief exposure (0.5 h) of $S1P_1$–EGFP-expressing astrocytes to either S1P (100 nM) or FTY720 (100 nM) induced rapid internalization of $S1P_1$ (Fig. 4*A*, 0.5 h). $S1P_1$ was recycled to the cell surface in S1P-treated astrocytes (Fig. 4*A*, 2 h). In sharp contrast, $S1P_1$ remained internalized in FTY720-treated astrocytes (Fig. 4*A*, 2 h). FTY720 can be endogenously phosphorylated by sphingo-

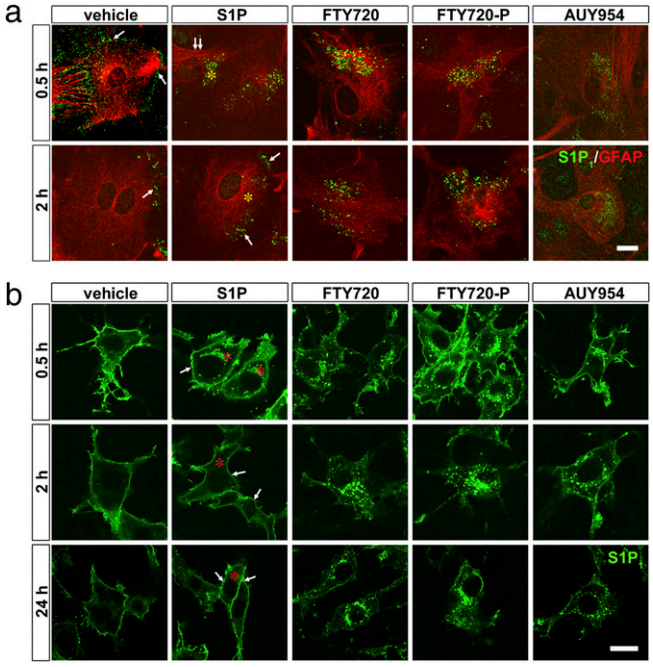

Fig. 4. Functional antagonism through prolonged $S1P_1$ internalization by S1P receptor agonists in astrocytes. (*A*) Primary cortical astrocytes from $S1P_1$ conditional null mutants (*S1pr1*$^{loxP/loxP}$; *nestin-cre*) were reconstituted by retroviral-mediated expression of a functional $S1P_1$–EGFP fusion protein. Cells were treated with vehicle (0.1% BSA), S1P (100 nM), FTY720 (100 nM), FTY720-P (100 nM), or AUY954 (100 nM). Cells were assessed to identify receptor internalization (0.5 h) or possible receptor recycling to the cell surface (2 h) after ligand exposure compared with vehicle controls. At 0.5 h after vehicle exposure, native EGFP fluorescence identified cell surface labeling; all other panels used anti-GFP immunofluorescence, which produced punctate labeling. (*B*) Functional antagonism through receptor internalization is maintained over extended periods (0.5, 2, and 24 h) in C6 glioma cells overexpressing $S1P_1$. At each time point, cells were fixed, and native EGFP fluorescence was detected. C6 glioma cells allowed extended examination periods of compound exposure compared with primary astrocytes. The sustained receptor internalization in both primary astrocytes and C6 cells is consistent with $S1P_1$ functional antagonism in the presence of FTY720, FTY720-P, and AUY954, 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.)

sine kinase 2 (Sphk2) (6), which is expressed in these astrocytes (Fig. S6*B*), consistent with $S1P_1$ effects activated by exogenously applied FTY720 or FTY720-P. As with FTY720, exposure to either FTY720-P or AUY954 also produced prolonged internalization of $S1P_1$ (Fig. 4*A*, 2 h). These data were recapitulated in the astrocyte-like glioma cell line, C6, using $S1P_1$ overexpression (Fig. 4*B*) 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 AUY954, similar to the results obtained in astrocytes.

Discussion

The aggregate results support functional antagonism of astrocyte $S1P_1$ rather than forms of agonism as the predominant receptor mechanism for FTY720 efficacy. Receptor internalization of FTY720-P-$S1P_1$ associated with a report of persistent agonism (10) is not likely to be relevant, because genetic as well as pharmacological loss of CNS $S1P_1$ 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 $S1P_1$ signaling may be relevant to the actions of FTY720 on lymphocyte egress (10); however, the separation of immunological and CNS $S1P_1$ 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 $S1P_1$ loss along with reduced cytokine expression and S1P levels identify possible downstream mechanisms activated by $S1P_1$ loss or FTY720 exposure. Other nonmutually exclusive mechanisms downstream of S1P signaling in astrocytes include important astrocyte effects on the blood–brain barrier or possible neuroprotective actions, all of which deserve further investigation. $S1P_1$ 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 $S1P_1$ 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 $S1P_1$ signaling from astrocytes (Fig. S7). Genetic, in situ hybridization, and receptor internalization data identify astrocytes as the primary CNS cell type affected by $S1P_1$ 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 in 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 conform to National Institutes of Health guidelines and public law. EAE was induced by MOG amino acid 35–55 (MEVGWYRSPFSRVVHLYRNGK, 98% purity; American Peptide) immunization in 7- to 9-wk-old female mice: WT mice (C57BL/6J) and conditional null mutants for $S1P_1$ in different CNS cell lineages, including *S1pr1*$^{loxP/loxP}$; *nestin-cre* ($S1P_1$ deletion in all CNS cell lineages) (23), *S1pr1*$^{loxP/loxP}$; *synapsin-cre* ($S1P_1$ deletion in neuronal lineages) (24), and *S1pr1*$^{loxP/loxP}$; *GFAP-cre* ($S1P_1$ deletion in astrocyte lineages) (25, 26) or littermate control mice (*S1pr1*$^{loxP/loxP}$). For immunization, MOG was dissolved in water, and an emulsion was prepared in Complete Freund's 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 (10 min) in 4% paraformaldehyde (PFA), permeabilized in 0.5% Triton X-100, and blocked with 3% BSA. Tissue sections were labeled with primary antibodies or antisera; mouse anti-GFAP (1:1,000; 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 unrecombined control and various $S1P_1$ 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-GFAP 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 system. S1P levels were calculated compared 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-1β 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 Luminex.

Determination of $S1P_1$ Internalization and Recycling in Astrocytes. Primary astrocytes were isolated from cerebral cortices of $S1P_1$ conditional KO mice and retrovirally transduced with an $S1P_1$–EGFP fusion construct. Cells were labeled with mouse anti-GFAP and rabbit anti-GFP and visualized with fluorescence-conjugated anti-IgG. $S1P_1$ 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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