British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01579.x
www.brjpharmacol.org

RESEARCH PAPER

Oral fingolimod rescues the functional deficits of synapses in experimental autoimmune encephalomyelitis

S Rossi[1,2]*, T Lo Giudice[1,2]*, V De Chiara[1,2], A Musella[1,2], V Studer[1,2], C Motta[1,2], G Bernardi[1,2], G Martino[3], R Furlan[3], A Martorana[1,2] and D Centonze[1,2]

[1]*Clinica Neurologica, Dipartimento di Neuroscienze, Università Tor Vergata, Rome, Italy,* [2]*Fondazione Santa Lucia/Centro Europeo per la Ricerca sul Cervello (CERC), Rome, Italy, and* [3]*Neuroimmunology Unit, Institute of Experimental Neurology (INSpe), Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy*

Correspondence
Diego Centonze, Clinica Neurologica, Dipartimento di Neuroscienze, Università Tor Vergata, Via Montpellier 1, 00133 Rome, Italy. E-mail: centonze@uniroma2.it

*S. R. and T. L. G. are equally contributing authors.

Keywords
EAE; EPSC; glutamate; multiple sclerosis; neurodegeneration; striatum

Received
7 March 2011
Revised
15 June 2011
Accepted
30 June 2011

BACKGROUND AND PURPOSE
Alterations of glutamate-mediated synaptic transmission occur early during neuroinflammatory insults, and lead to degenerative neuronal damage in multiple sclerosis (MS) and also in experimental autoimmune encephalomyelitis (EAE), which is a murine model of MS. Fingolimod is an effective orally active agent for the treatment of MS, affecting lymphocyte invasion of the brain. However, it is still unclear if fingolimod can be neuroprotective in this disorder.

EXPERIMENTAL APPROACH
Using neurophysiological recordings and morphological evaluation of dendritic integrity, we evaluated the effects of oral fingolimod on the clinical score of EAE mice in order to determine whether the compound was associated with preservation of synaptic transmission.

KEY RESULTS
Oral fingolimod prevented and reversed the pre- and postsynaptic alterations of glutamate transmission in EAE mice. These effects were associated with a clear amelioration of the clinical deterioration seen in EAE mice, and with a significant inhibition of neuronal dendritic pathology. Fingolimod did not alter the spontaneous excitatory postsynaptic currents in control animals, suggesting that only the pathological processes behind the inflammation-induced defects in glutamate transmission were modulated by this compound.

CONCLUSIONS AND IMPLICATIONS
The beneficial effects of fingolimod on the clinical, synaptic and dendritic abnormalities of murine EAE might correlate with the neuroprotective actions of this agent, as observed in MS patients.

LINKED ARTICLE
This article is commented on by Gillingwater, pp. 858–860 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01612.x

Abbreviations
ACSF, artificial cerebrospinal fluid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EAE, experimental autoimmune encephalomyelitis; EPSC, excitatory postsynaptic current; MS, multiple sclerosis; S1P, sphingosine-1-phosphate

Introduction

The clinical course of multiple sclerosis (MS) in most patients is characterized by recurrent inflammatory episodes in the early stages (relapsing-remitting MS), and by a progressive neurological decline in the late phases (secondary progressive MS). A minority of patients experience a progressive course of the disease demonstrable immediately after the onset of the disease (primary progressive MS) (Compston and Coles, 2008; Trapp and Nave, 2008). Inflammation-triggered neurodegenerative damage is ultimately responsible for the primary and secondary progressive phases of MS, and this damage is particularly refractory to currently approved immunomodulatory or immunosuppressant therapies (Amor *et al.*, 2010; Dutta and Trapp, 2010; Bates, 2011).

Fingolimod is an effective orally active agent for the treatment of MS, which is able to interfere with the inflammatory phase of the disease through the prevention of lymphocyte invasion of the CNS (Matloubian *et al.*, 2004; Kataoka *et al.*, 2005; Pappu *et al.*, 2007; Brinkmann *et al.*, 2010; Cohen *et al.*, 2010; Kappos *et al.*, 2010). The compound is a structural analogue of a natural sphingosine (Chun and Hartung, 2010) and exerts its biological effects through the modulation of sphingosine-1-phosphate (S1P) receptors (receptor nomenclature follows Alexander *et al.*, 2011), after being phosphorylated *in vivo* by the ubiquitously expressed sphingosine kinase 2 (Billich *et al.*, 2003).

Evidence is also accumulating that fingolimod might have a neuroprotective effect in MS and in the rodent model, experimental autoimmune encephalomyelitis (EAE) (Balatoni *et al.*, 2007; Foster *et al.*, 2007; Dev *et al.*, 2008; Miron *et al.*, 2008). Based on these observations, a clinical trial is ongoing to investigate the effect of fingolimod in primary progressive forms of MS (Clinical Trials.gov Identifier: NCT00731692).

The cellular and molecular determinants of the neurodegenerative damage in MS are poorly understood, but evidence suggests that deficits in central synaptic transmission might be involved (Pitt *et al.*, 2000; Smith *et al.*, 2000; Zhu *et al.*, 2003; Rossi *et al.*, 2010; Ziehn *et al.*, 2010). By means of neurophysiological recordings from single neurons, we have recently found in mice with EAE that central neurons prone to degenerate in MS undergo dramatic changes in glutamate-mediated transmission, starting in the presymptomatic phase of the disease and evolving independently of demyelination or axonal injury (Centonze *et al.*, 2009; Rossi *et al.*, 2011). These alterations involve both the pre- and the postsynaptic compartment of excitatory synapses, resulting in increased glutamate release (Rossi *et al.*, 2010) and increased glutamate AMPA receptor sensitivity (Centonze *et al.*, 2009). These synaptic deficiencies are reliable correlates of the degenerative damage seen in EAE, because they are associated with marked dendritic pathology, and can be rescued by pharmacological blockade of glutamate AMPA receptors (Centonze *et al.*, 2010).

In the present investigation, we explored the effects of fingolimod on the clinical, synaptic and dendritic abnormalities of EAE mice in order to provide a possible correlate of the neuroprotective action of this agent.

Methods

EAE induction and clinical score

All animal care and experimental procedures were in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of mice used. All animals were housed, four per cage with food and water *ad libitum*, on a 12 h light/dark cycle with lights on at 06:00 h and controlled (22–23°C) temperature.

The experimental procedure has been described previously (Centonze *et al.*, 2009; Rossi *et al.*, 2010; 2011). Chronic, relapsing EAE was induced in C57BL/6 female mice (n = 45, 8 weeks old) by subcutaneous immunization with 300 µL of 200 µg myelin oligodendrocyte glycoprotein peptide 35–55 (MOG 35–55) (Multiple Peptide System) in incomplete Freund's adjuvant containing 8 mg·mL^{-1} *Mycobacterium tuberculosis* (strain H37Ra; Difco). *Pertussis* toxin (Sigma) (500 ng) was injected on the day of the immunization and again 2 days later. Body weight and clinical score (0 = healthy; 1 = limp tail; 2 = ataxia and/or paresis of hindlimbs; 3 = paralysis of hindlimbs and/or paresis of forelimbs; 4 = tetraparalysis; 5 = moribund or death) were recorded daily, by an experimenter unaware of the assigned treatments. Mice treated with complete Freund's adjuvant without MOG, and *Pertussis* toxin were used as healthy control group (n = 20).

Fingolimod, supplied by Novartis Pharma AG, was given once daily by gavage at a dose of 0.3 mg·kg^{-1} body weight. For prophylactic or therapeutic treatments, oral dosing started on the same day as the immunization or at the peak of the disease (20 days post-immunization, dpi) respectively.

Electrophysiology

Mice were killed by cervical dislocation under halothane anaesthesia, and corticostriatal coronal slices (200 µm) were prepared from fresh tissue blocks of the brain with the use of a vibratome (Centonze *et al.*, 2009; Rossi *et al.*, 2010). A single slice was then transferred to a recording chamber and submerged in a continuously flowing artificial CSF (ACSF) (34°C, 2–3 mL·min^{-1}) gassed with 95% O_2-5% CO_2. The composition of the control ACSF was (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 glucose, 25 $NaHCO_3$.

Recording pipettes were advanced towards individual striatal cells in the slice under positive pressure and visual control (WinVision 2000, Delta Sistemi, Italy) and, on contact, tight GΩ seals were made by applying negative pressure. The membrane patch was then ruptured by suction and membrane current and potential monitored using an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell access resistances measured in voltage clamp were in the range of 5–20 MΩ.

Whole-cell patch clamp recordings were made with borosilicate glass pipettes (1.8 mm o.d.; 2–3 MΩ), in voltage-clamp mode, at the holding potential of –80 mV. To study spontaneous glutamate-mediated excitatory postsynaptic currents (sEPSCs), the recording pipettes were filled with internal solution of the following composition (mM): K^+-gluconate (125), NaCl (10), $CaCl_2$ (1.0), $MgCl_2$ (2.0), 1,2-bis (2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid (BAPTA; 0.5), HEPES (19), GTP; (0.3), Mg-ATP; (1.0),

adjusted to pH 7.3 with KOH. Bicuculline (10 μM) was added to the perfusing solution to block $GABA_A$-mediated transmission.

Synaptic events were stored by using P-CLAMP 9 (Axon Instruments) and analysed off line on a personal computer with Mini Analysis 5.1 (Synaptosoft, Leonia, NJ, USA) software. The detection threshold of sEPSCs was set at twice the baseline noise. No false events were identified, and this was confirmed by visual inspection for each experiment. Offline analysis was performed on spontaneous synaptic events recorded during fixed time epochs (1–2 min, three to five samplings), sampled every 5 or 10 min. Only cells that exhibited stable frequencies in control (less than 20% changes during the control samplings) were used for analysis. For kinetic analysis, events with peak amplitude between 10 and 50 pA were grouped, aligned by half-rise time, normalized by peak amplitude and averaged to obtain rise times and decay times. Events with complex peaks were eliminated.

One to six cells per animal were recorded. For each type of experiment and time point, at least four distinct animals were employed from each experimental group. Throughout the text '*n*' refers to the number of cells, unless otherwise specified. Drugs were applied by dissolving them to the desired final concentration in the bathing ACSF.

Golgi staining

The single-section Golgi technique was carried out on coronal sections (100 μm) containing striata from healthy mice ($n = 5$), untreated EAE mice (20 dpi, $n = 5$) and EAE mice treated with prophylactic fingolimod (20 dpi, $n = 5$). Mice were anaesthetized with chloral hydrate (400 $mg \cdot kg^{-1}$ i.p.), perfused through the ascending aorta with a solution of NaCl 0.9% for 5 min, and then perfused with 3% paraformaldehyde with 0.4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Sections from animals perfused with glutaraldehyde were treated with sodium borohydrate (Sigma, Italy) 0.1% in PBS 0.01 M pH 7.4. Brains were removed and coronal sections were cut on a vibrating microtome and collected in phosphate buffer 0.1 M pH 7.4 at 4°C for subsequent section Golgi impregnation. Sections were post-fixed in 1% osmium tetroxide for 20 min and were free floated in 3.5% aqueous solution of potassium dichromate for 12 h. Then, each section was placed flat on a microscope slide and excess of potassium dichromate was blotted from the edge of the tissue section. Each section was sandwiched with another microscope slide, and the entire assembly was placed in 1–2% of silver nitrate solution overnight. The microscope slide was then carefully separated and the Golgi-impregnated section was dehydrated, softened with cedar wood oil and mounted under coverglass. A set of five coronal sections from each animal was selected for spine density counts. Three areas (one dorsallateral, one in the middle and one ventral lateral) of both striata were analysed.

The spine density of striatal neurons was calculated by counting the spines along the dendritic length from soma up to 100 μm, by using a 40X oil immersion lens. Only completely impregnated dendrites found entirely within the tissue section were used for spine counts ($n = 100$ dentrites for mice).

Statistical analysis

Results are shown as the mean ± SE. Multiple comparisons were analysed by ANOVA followed by Tukey honestly significant difference in the case of parametric data or by Kruskal–Wallis test followed by Dunn's test in the case of non parametric data. Comparisons between two groups were analysed by paired or unpaired Student's *t*-test or Mann–Whitney. The significance level was established at $P < 0.05$.

Materials

Drugs were supplied as follows: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and MK-801 from Tocris Cookson, Bristol, UK; bepridil and bicuculline from Sigma-RBI, St. Louis, MO; FTY720-P from Novartis Pharma AG, Basel.

Results

Effect of fingolimod on the clinical score of EAE mice

All EAE mice from the untreated control group ($n = 28$ mice) developed neurological signs around dpi 12, reaching a maximal level on dpi 20 (2.8 ± 0.5). The severity of these neurological manifestations was less pronounced at dpi 50 (2.0 ± 0.3). The elevation of EAE scores was significantly inhibited in the EAE group treated with prophylactic fingolimod (score at dpi 12: 0, score at dpi 20: 0.27 ± 0.12, score at dpi 50: 0.13 ± 0.09; $n = 15$, $P < 0.01$ compared with the EAE untreated group) (Figure 1A).

We also evaluated the therapeutic effect of fingolimod on the chronicity of EAE. In these experiments, a group of EAE mice was randomly divided into two subgroups consisting of 10 mice each, and fingolimod or vehicle were administered daily from dpi 20. EAE-associated clinical signs were decreased by dpi 24 in the treated group (Figure 1B).

Effect of fingolimod on the kinetic properties of sEPSCs in EAE

Our previous studies have shown (Centonze *et al.*, 2009) that the duration of glutamate-mediated sEPSCs was increased in striatal neurons of untreated EAE mice. A slower decay time accounted for increased sEPSC duration. This alteration was evident both in the presymptomatic (7 dpi; $P < 0.01$), acute (20 dpi; $P < 0.01$) and chronic phases of EAE (50 dpi; $P < 0.01$) (n = at least 20 neurons for both experimental groups). Prophylactic treatment with fingolimod completely prevented the alteration of sEPSC shape. In fact, sEPSC decay times were not different from those in control mice (7 dpi; $P > 0.05$, 20 dpi; $P > 0.05$, 50 dpi; $P > 0.05$; (n = at least 20 neurons for each experimental group) (Figure 2A). Rise time of sEPSCs was not affected by EAE induction and fingolimod treatment (Figure 2B).

Therapeutic intervention with fingolimod, starting on dpi 20, not only reversed the clinical pathology of EAE mice, but also restored the alterations of sEPSC duration of EAE mice. Treated animals, in fact, exhibited a normalization of sEPSC duration at 50 dpi ($n = 15$, $P > 0.05$ from control), while the untreated animals continued to exhibit clear sEPSC decay time prolongation ($n = 13$; $P < 0.01$ from control) (Figure 2C).

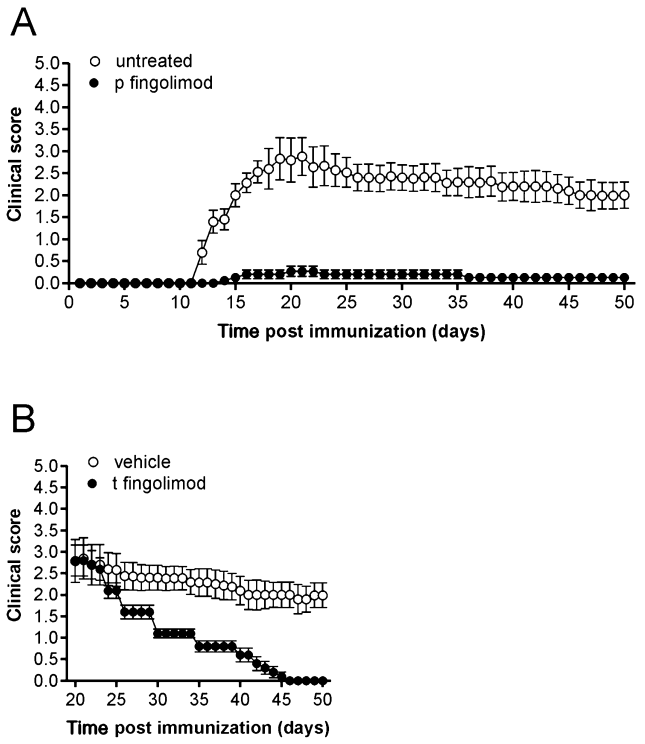

Figure 1

Effect of fingolimod on the clinical score of EAE mice. (A) The elevation of EAE scores was significantly inhibited in the EAE group treated with prophylactic fingolimod (p fingolimod, by gavage at a dose of 0.3 mg·kg^{-1} body weight, $n = 15$; untreated $n = 28$). (B) The graph shows that fingolimod administered daily since dpi 20 (therapeutic treatment, t fingolimod, $n = 10$; vehicle treated $n = 10$) reversed EAE-associated clinical signs.

As previously reported (Centonze *et al.*, 2009; Rossi *et al.*, 2010), sEPSCs recorded from both control and EAE mice were abolished in the presence of the glutamate AMPA receptor antagonist CNQX ($n = 6$ for both conditions), indicating that these synaptic events were entirely mediated by these receptor subtypes (not shown).

Effect of fingolimod on the frequency of sEPSCs in EAE

The duration and frequency of sEPSCs is increased in EAE mice (Centonze *et al.*, 2009; Rossi *et al.*, 2010) which is expected with abnormalities in both pre- and postsynaptic glutamatergic transmission. In agreement with previous reports (Centonze *et al.*, 2009; Rossi *et al.*, 2010), the frequency of glutamate-mediated sEPSCs in our model was increased in the presymptomatic (7 dpi; $P < 0.05$) and acute (20 dpi; $P < 0.05$) phases of EAE, whilst it was normal in the chronic stages (50 dpi; $P > 0.05$) (Figure 3A). In accordance with the data on the kinetic properties of sEPSCs, sEPSC frequency recorded at dpi 7, dpi 20, and dpi 50 from EAE mice pre-treated with fingolimod was also normal (n = at least 20 neurons for each experimental group) (Figure 3A).

Overactive NMDA receptors of the presynaptic terminal (Centonze *et al.*, 2009) and abnormal functioning of axonal

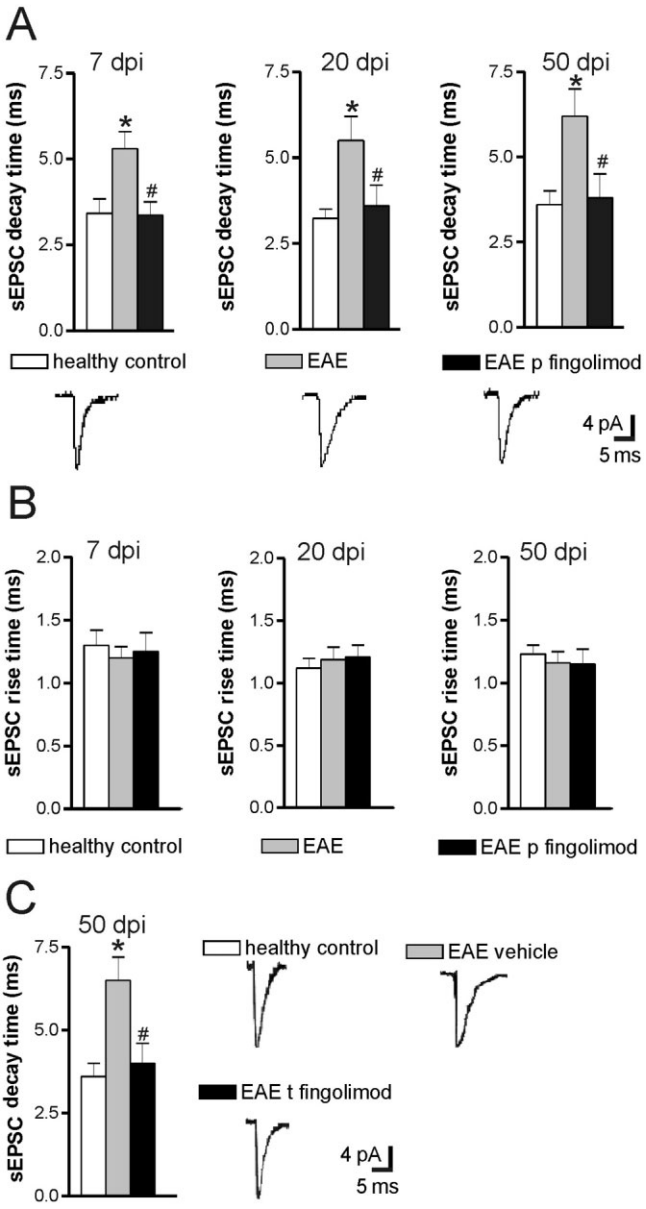

Figure 2

Effect of fingolimod on the kinetic properties of sEPSCs in EAE. (A) The histograms show that prophylactic treatment with fingolimod completely prevented the alteration of sEPSC decay time in presymptomatic, acute and chronic phase of EAE. At least twenty neurons from at least four mice in each experimental group were used for the analyses. The electrophysiological traces are examples of sEPSC mean peak obtained by group analysis from neurons of healthy control, untreated EAE and prophylactic fingolimod-treated EAE mice at 20 dpi. (B) Rise time of sEPSCs was not affected by EAE induction and fingolimod treatment. (C) Therapeutic treatment with fingolimod reversed the decay time prolongation of sEPSCs recorded during chronic phase of EAE. Healthy control: $n = 21$ cells; EAE vehicle: $n = 13$ cells; EAE t fingolimod: $n = 15$ cells. The electrophysiological traces are examples of sEPSC mean peak obtained by group analysis from neurons of healthy control, untreated EAE and therapeutic fingolimod-treated EAE mice at 50 dpi. *$P < 0.05$ significantly different from control, #$P < 0.05$ significantly different from untreated EAE; ANOVA followed by Tukey honestly significant difference test.

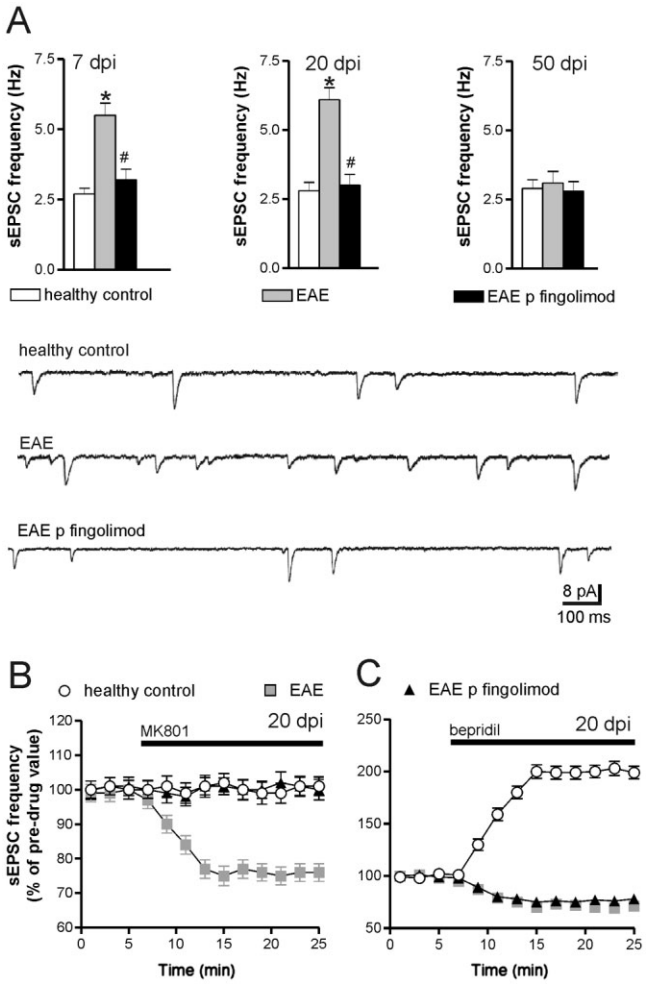

Figure 3

Effect of fingolimod on the frequency of sEPSCs in EAE. (A) The histograms show that the frequency of sEPSCs was increased in the presymptomatic and acute phase of EAE but normal in EAE mice treated with prophylactic fingolimod. At least twenty neurons from at least four mice in each experimental group were used for the analyses. The electrophysiological traces are examples of sEPSCs (downward deflections) recorded from striatal neurons of healthy control, untreated EAE and prophylactic fingolimod-treated EAE mice at 20 dpi. (B) The graph shows that MK-801, an NMDA receptor antagonist, reduced sEPSC frequency in untreated EAE mice but not in control and in fingolimod-treated EAE mice. Healthy control: n = 10 cells; EAE: n = 16 cells; EAE p fingolimod: n = 11 cells; paired t-test. (C) Bepridil, a blocker of Na^+/Ca^{++} exchanger, caused a paradoxical inhibition of sEPSC frequency in both untreated and fingolimod-treated EAE mice. Healthy control: n = 6 cells; EAE: n = 10 cells; EAE p fingolimod: n = 8 cells; paired t-test. *P < 0.05 significantly different from control, #P < 0.05 significantly different from untreated EAE; ANOVA followed by Tukey honestly significant difference test.

Na^+/Ca^{++} exchangers (Craner *et al.*, 2004; Rossi *et al.*, 2010) have been proposed to account for increased glutamate release and sEPSC frequency in EAE. Thus, we also tested the sensitivity of glutamate synapses to the NMDA receptor antagonist MK-801 and to bepridil, a blocker of the Na^+/Ca^{++} exchanger, in control EAE mice or EAE mice treated with fingolimod. As previously reported (Centonze *et al.*, 2009), MK-801 significantly reduced sEPSC frequency (n = 16, P < 0.05 with respect to pre-drug values) in the acute phase of EAE (20 dpi) but not in control mice (n = 10, P > 0.05 with respect to pre-drug values). In mice treated with fingolimod the sensitivity of glutamate synapses to MK-801 was similar to control mice (P > 0.05, n = 11) (Figure 3B).

Again, as previously reported (Rossi *et al.*, 2010), bepridil increased the frequency of sEPSCs (n = 6) in control mice (P < 0.01), while it caused a paradoxical inhibition of sEPSC frequency in untreated EAE mice (20–30 dpi; n = 10, P < 0.05). Fingolimod failed to normalize the activity of the Na^+/Ca^{++} exchanger in EAE mice, because bepridil reduced sEPSC frequency also in EAE mice pre-treated with fingolimod (20–30 dpi; n = 8, P < 0.05 with respect to pre-drug values, P > 0.05 respect to untreated EAE group) (Figure 3C).

Effect of fingolimod on basal synaptic transmission

The data from experiments detailed above might indicate that fingolimod alters sEPSCs in EAE mice because it modulates basal glutamate transmission at central synapses. To address this possibility, we tested, *in vitro*, the effect of the active metabolite of fingolimod, FTY720-P, applied in the bathing solutions of corticostriatal slices of control mice. In fact, fingolimod is phosphorylated *in vivo*, and the resulting metabolite, a ligand of S1P receptors, is responsible for the biological effects of the compound (Billich *et al.*, 2003; Chun and Hartung, 2010). FTY720-P failed to alter frequency (99 ± 3%; Figure 4A), amplitude (97 ± 3%; Figure 4B) and kinetic properties (rise time: 101 ± 1%; decay time: 100 ± 1%; Figure 4C) of sEPSCs recorded from control neurons (n = at least 10 neurons for each parameter, P > 0.05 for each parameter compared with pre-drug values), indicating that fingolimod corrected the synaptic alterations induced by EAE, but it did not interfere with physiological synaptic transmission.

Effect of fingolimod on dendritic spine loss in EAE

We next investigated the effects of fingolimod on EAE-induced loss of dendritic spines in striatal neurons at the acute phase of the disease. Single-section Golgi preparations showed that second dendritic branches of striatal neurons had a marked reduction of spine number along the whole extent of the dendrites in EAE (20 dpi, n = 5, 17 ± 2, P < 0.01) compared with normal animals (n = 5, 30 ± 3), although cell somata and primary dendrites were similar in the two groups. EAE mice receiving fingolimod (20 dpi, n = 5) exhibited the same unaltered morphological features of the somata of striatal neurons, but presented increased spines compared with untreated EAE mice (spine number/100 μm: 28 ± 3, P < 0.01) (Figure 5).

Discussion and conclusions

The present study demonstrated that the clinical, synaptic and neuropathological defects of EAE mice are significantly attenuated by oral fingolimod, suggesting that treatment with this pharmacological agent could be neuroprotective in

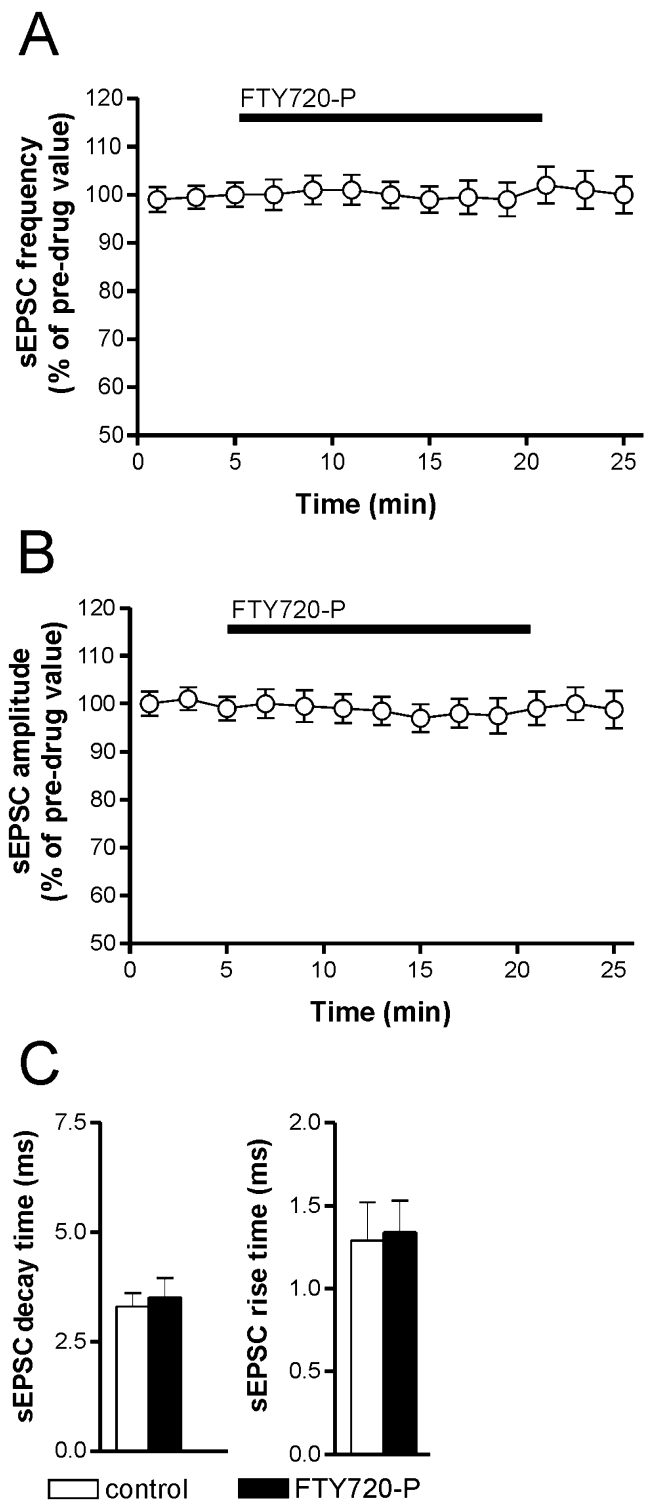

Figure 4

Effect of fingolimod on basal synaptic transmission. (A–C) FTY720-P failed to alter the frequency (A; $n = 15$ cells for both groups; paired *t*-test), the amplitude (B; $n = 15$ cells for both groups; paired *t*-test) and the kinetic properties (C; control: $n = 11$ cells; FTY720-P: $n = 12$ cells; unpaired *t*-test) of sEPSCs recorded from control neurons.

patients with MS. Overactive NMDA receptors of the presynaptic terminal and sensitized AMPA receptors of postsynaptic terminal were normalized by oral fingolimod. However, the abnormal functioning of the axonal Na^+/Ca^{++} exchanger was unaffected by the treatment, suggesting that the synapse is specifically protected. Furthermore, the fact that not all the electrophysiological alterations induced by EAE were prevented by fingolimod might suggest that this effect is not a mere consequence of protection against EAE induction, but a specific neuroprotection. In addition, we have shown that oral fingolimod clearly rescues the functional deficiencies of the synapses, but further molecular immunolocalization studies will be necessary to assess the structural protection of the synapses.

Fingolimod did not alter glutamate transmission in control animals, indicating that only the pathological process behind the inflammation-induced sEPSC alterations was modulated by this compound and physiological transmission was unaffected by fingolimod.

Based on a series of experimental findings, excessive signalling at glutamate synapses has been proposed as a crucial intermediate step linking inflammation and neurodegeneration in EAE and in MS (Centonze *et al.*, 2010). Glutamate levels are in fact higher in the cerebrospinal fluid (Stover *et al.*, 1997; Sarchielli *et al.*, 2003) and in the brains of MS patients (Srinivasan *et al.*, 2005; Cianfoni *et al.*, 2007), whilst AMPA, NMDA and kainate receptors are up-regulated (Newcombe *et al.*, 2008). Also the expression of glutamate transporters is abnormal in MS (Geurts *et al.*, 2003; 2005; Vallejo-Illarramendi *et al.*, 2006) and EAE (Hardin-Pouzet *et al.*, 1997; Ohgoh *et al.*, 2002), and the loss of glutamate transporters in cortical lesions correlates with microglial activation and synaptic damage (Vercellino *et al.*, 2007). Furthermore, overactivation of glutamate receptors causes MS-like lesions (Matute *et al.*, 2001), whereas glutamate receptor antagonists exert beneficial effects in EAE (Wallström *et al.*, 1996; Bolton and Paul, 1997; Pitt *et al.*, 2000; Smith *et al.*, 2000; Centonze *et al.*, 2009) and in MS (Plaut, 1987). We have recently found that *in vivo* blockade of AMPA receptors not only ameliorated the clinical course of EAE, but also preserved dendritic spine loss which occurs in this model of MS. These results support the involvement of AMPA receptors in EAE-induced dendritic spine degeneration (Centonze *et al.*, 2009).

The mechanisms by which effector T lymphocytes, activated in the periphery against myelin antigens, lead to the synaptic alterations and dendritic pathology in the CNS of EAE mice has been partially elucidated in a previous study. We have proposed that the inflammatory infiltrates composed of $CD3^+$ T cells and found in the white as well as in the grey matter of EAE mice (Centonze *et al.*, 2009), represent the first step in a chain of events culminating in synaptic alterations, because preparations of activated T lymphocytes from EAE mice were able to increase the duration of sEPSCs when placed onto brain slices of normal mice, thus replicating the synaptic defects of EAE (Centonze *et al.*, 2010). Microglial cells are also involved in this pathological process because they were found to be massively activated in the presymptomatic and acute phases of EAE in response to pro-inflammatory cytokines released from T lymphocytes, and because they too were able to mimic the effects of EAE on sEPSCs through the release of TNF-α. TNF-α, in turn, altered

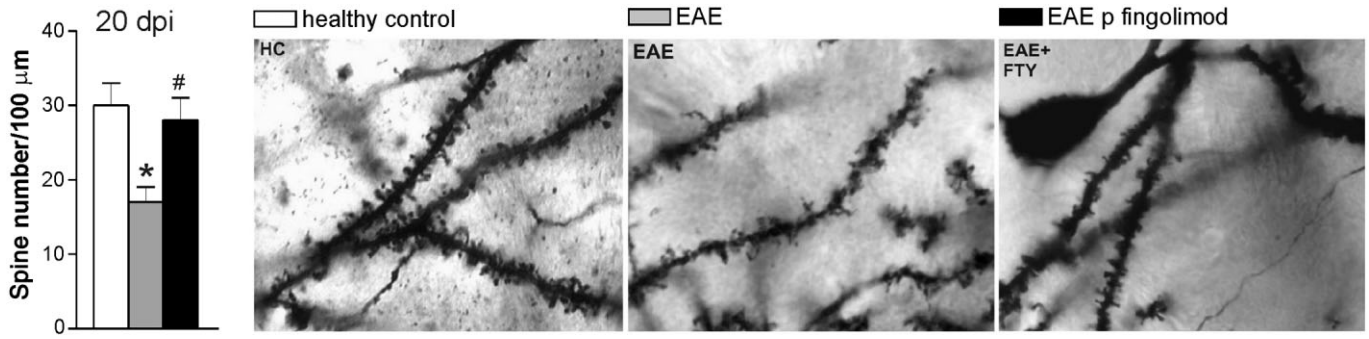

Figure 5

Effect of fingolimod on dendritic spine loss in EAE. Prophylactic fingolimod treatment significantly reduced the dendritic spine loss observed during acute phase of EAE (expressed as spine density: number per 100 μm). Examples of single-section Golgi preparations from healthy control (n = 5 mice), untreated EAE (n = 5 mice) and prophylactic fingolimod-treated EAE mice (n = 5 mice) at 20 dpi. *P < 0.05 significantly different from control, #P < 0.05 significantly different from untreated EAE. Kruskal–Wallis test followed by Dunn's test.

sEPSCs by increasing expression and phosphorylation of glutamate AMPA receptors in the postsynaptic membranes, and the resulting neuronal overstimulation activated a localized apoptotic cascade leading to dendritic spine loss and finally neuronal deafferentation (Centonze *et al.*, 2009).

The effects of fingolimod on the synaptic hyperexcitability accompanying EAE might occur at various levels of the inflammation-synaptic degeneration pathway, including T-cell invasion of the grey matter, microglial activation, TNF-α release and neuronal damage. Fingolimod limits activated T lymphocyte entry into the CNS via sequestration into secondary lymph nodes (Matloubian *et al.*, 2004; Kataoka *et al.*, 2005; Pappu *et al.*, 2007; Brinkmann *et al.*, 2010; Cohen *et al.*, 2010; Kappos *et al.*, 2010), and by decreasing the permeability of the blood-brain barrier (Brinkmann *et al.*, 2004; Miron *et al.*, 2008). But, by virtue of its high lipophilicity, fingolimod readily penetrates the brain and influences the activity of glial cells and of neurons (Dev *et al.*, 2008; Brinkmann, 2009; Lee *et al.*, 2010). Lymphocytes, and many CNS resident cells express S1P receptors, and thus respond to fingolimod (Sim-Selley *et al.*, 2009; Nishimura *et al.*, 2010; Choi *et al.*, 2011). The CNS-directed actions of fingolimod are thought to play a significant role in the beneficial effects of the compound in EAE and direct intracerebroventricular administration of fingolimod ameliorates the clinical course of the disease (Schubart *et al.*, 2007; Miron *et al.*, 2008).

Fingolimod inhibits the activity of microglial cells by altering cytokine production (Tham *et al.*, 2003). This effect might be potentially relevant for the synaptic effects described here, because TNF-α release from microglia has been implicated in the alterations of sEPSCs of EAE mice (Centonze *et al.*, 2009). In addition, SIP receptors have also been reported to influence neuronal excitability (MacLennan *et al.*, 2001; Zhang *et al.*, 2006) and neurite extension (Toman *et al.*, 2004), providing a possible additional mechanism by which fingolimod protects excitatory transmission and dendritic arborization in EAE.

In conclusion, further work is required to clarify the mechanisms of fingolimod effects on synaptic and neuronal function during EAE. This information might be helpful to define the significance of this agent as a neuroprotective compound in MS, as well as in other CNS disorders in which inflammation-driven neurodegeneration plays a role.

Acknowledgement

This study was supported by a grant from Novartis Pharma to D. C. and R. F.

Conflicts of interest

Silvia Rossi received honoraria for writing from Bayer Schering and funding for travelling from Novartis, Teva, Merck Serono. She is involved as sub-investigator in clinical trials for Novartis, Merck Serono, Teva, Bayer Schering, Sanofi-aventis, Biogen Idec.

Valentina De Chiara received funding for travelling by Teva. She is involved as study coordinator in clinical trials for Novartis, Merck Serono, Teva, Bayer Schering, Sanofi-Aventis, Biogen Idec.

Giorgio Bernardi is the principal investigator in clinical trials for Merck Serono and Teva.

Diego Centonze is an Advisory Board member of Merck-Serono, Teva, Bayer Schering, and received funding for travelling and honoraria for speaking or consultation fees from Merck Serono, Teva, Novartis, Bayer Schering, Sanofi-aventis, Biogen Idec. He is also an external expert consultant of the European Medicine Agency (EMA), and the principal investigator in clinical trials for Novartis, Merck Serono, Teva, Bayer Schering, Sanofi-aventis, Biogen Idec.

The other authors have no conflict of interests to declare.

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