

ORIGINAL ARTICLE

Antidepressant activity of fingolimod in mice

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Abstract

Recent findings indicate that fingolimod, the first oral drug approved for the treatment of multiple sclerosis (MS), acts as a direct inhibitor of histone deacetylases (HDACs) and enhances the production of brain-derived neurotrophic factor (BDNF) in the CNS. Both mechanisms are relevant to the pathophysiology and treatment of major depression. We examined the antidepressant activity of fingolimod in mice subjected to chronic unpredictable stress (CUS), a model of reactive depression endowed with face and pharmacological validity. Chronic treatment with fingolimod (3 mg kg⁻¹, i.p., once a day for 4 weeks) reduced the immobility time in the forced swim test (FST) in a large proportion of CUS mice. This treatment also caused anxiogenic-like effects in the social interaction test without affecting anxiety-like behavior in the elevated plus maze or spatial learning in the water maze. CUS mice showed reduced BDNF levels and enhanced HDAC2 levels in the hippocampus. These changes were reversed by fingolimod exclusively in mice that showed a behavioral response to the drug in the FST. Fingolimod treatment also enhanced H3 histone K14-acetylation and adult neurogenesis in the hippocampus of CUS mice. Fingolimod did not affect most of the parameters we have tested in unstressed control mice. The antidepressant-like activity of fingolimod was confirmed in mice chronically treated with corticosterone. These findings show for the first time that fingolimod exerts antidepressant-like effect acting in a “disease-dependent” manner, and raise the interesting possibility that the drug could relieve depressive symptoms in MS patients independently of its disease-modifying effect on MS.

Abbreviations

BDNF, brain-derived neurotrophic factor; BrdU, 5-Br-2'-deoxyuridine; CUS, chronic unpredictable stress; DCX, doublecortin; EPM, elevated plus maze; FST, forced swim test; H3K14Ac, H3 histone acetylated on Lys14; HDAC, histone deacetylase; MS, multiple sclerosis; MWM, morris water maze; S1PR, sphingosine-1-phosphate receptor; SI, social interest; SPT, sucrose preference test; SR, social recognition.

Introduction

Multiple sclerosis (MS) is an immune-mediated disease that affects the white matter of the CNS and causes

demyelination, axonal degeneration, and neuronal death. Optic neuritis, pyramidal tract symptoms, and cerebellar dysfunction are hallmark features of MS. However, psychiatric symptoms such as depressed mood, anhedonia,

anxiety, and sleep disturbances may be present, and sometimes match the criteria for a comorbid diagnosis (Compston and Coles 2008).

Depression shows a high prevalence (up to 50%) in MS patients, and may worsen the course of MS by reducing the compliance to medication (D'Alisa et al. 2006; Feinstein 2007, 2011; Minden et al. 2014). Depression might develop in MS patients as a result of neuroinflammation or axonal damage in brain circuits involved in mood control (Miller et al. 2009; McNamara and Lotrich 2012; Felger and Lotrich 2013), or, more simply, might be a consequence of the poor quality of life and the awareness of a progressive neurological impairment in MS patients. In addition, interferon- β (IFN- β), which is a first-line drug in the treatment of MS, may cause depression *de novo* or worsen preexisting depression (Patten et al. 2005).

Most disease-modifying drugs used in the treatment of MS act preferentially outside the CNS to restrain autoimmunity (Comi 2013). Fingolimod, the first oral drug approved for the treatment of relapsing-remitting MS, causes immune suppression by inhibiting the egress of certain populations of T lymphocytes from secondary lymphoid organs (Aktas et al. 2010; Brinkmann et al. 2010; Pelletier and Hafler 2012). However, a growing body of evidence suggests that fingolimod may also act on neurons and glial cells resident in the CNS (Brinkmann et al. 2010; Pelletier and Hafler 2012; di Nuzzo et al. 2014). Fingolimod is a highly lipophilic sphingosine analog, which is transformed into the active metabolite, fingolimod-P, by intracellular type-2 sphingosine kinase. Fingolimod-P activates four types of membrane sphingosine-1-phosphate receptors (S1PR1, -3, -4, and -5), acting as a "superagonist" of S1P1R. Overactivation of S1P1R causes receptor internalization resulting into functional antagonism (Brinkmann et al. 2010).

Recent data indicate that fingolimod-P may also act in the cell nucleus to stimulate histone acetylation and gene expression *via* a direct inhibition of class-I histone deacetylases (HDACs) (Hait et al. 2014). This epigenetic mechanism provides a potential link between fingolimod and depression because HDAC inhibitors are known to produce antidepressant-like effects (Sun et al. 2013), and chronic social defeat stress causes a persistent decrease in H3 histone K14-acetylation (H3K14Ac) in the hippocampus (Covington et al. 2011).

Another link with depression is the ability of fingolimod to enhance the production of brain-derived neurotrophic factor (BDNF) in neurons (Deogracias et al. 2012; Doi et al. 2013; Fukumoto et al. 2014; Hait et al. 2014). BDNF levels are reduced in the hippocampus of mice exposed to acute or chronic stress (Nibuya et al. 1995; Barrientos et al. 2003), and in the hippocampus and

peripheral blood of depressed patients (Shimizu et al. 2003; Karge et al. 2005; Sen et al. 2008). In addition, an impairment of BDNF signaling in the hippocampus results into a depressive-like phenotype (Monteggia 2007; Taliaz et al. 2010), whereas increases in hippocampal BDNF levels cause antidepressant-like effect (Shirayama et al. 2002; Hoshaw et al. 2005; Krishnan and Nestler 2010).

From a therapeutic standpoint, it would be important to examine the antidepressant-like effect of fingolimod in mice developing experimental autoimmune encephalomyelitis (EAE), which models MS. However, this is an impossible task because the severe motor impairment associated with EAE precludes the analysis of depressive-like behavior. Thus, we decided to test the antidepressant-like activity of fingolimod using mice exposed to chronic unpredictable stress (CUS), which models reactive depression. We also examined the action of fingolimod in a second mouse model of depression based on chronic systemic treatment with corticosterone.

Materials and Methods

Animals and drugs

Six-week old C57BL/6J male mice were purchased from Harlan Laboratories (Italy). Animals were housed four per cage under standard conditions, with access to food and water ad libitum and a 12 h light/dark cycle (light on at 07:00 AM). Experimental procedures were carried out according to the European (86/609/EEC) and Italian (D. Lgs 116/92) guidelines of animal care. All efforts were made to minimize the number of animals used and their suffering. The experimental protocol was approved by the Italian Ministry of Health. Fingolimod hydrochloride (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, hydrochloride) was purchased by CABRU (Arcore, Italy), and dissolved in saline. Corticosterone was purchased from Sigma Aldrich (Milano, Italy).

Experimental design

We have tested the antidepressant-like activity of fingolimod using two established models of depression in mice: (i) exposure to CUS; and (ii) chronic administration of corticosterone. In the CUS paradigm, we used two sets of mice, each including the following groups: (i) unstressed mice treated i.p. with saline or fingolimod (3 mg kg⁻¹) for 4 weeks; and (ii) mice exposed to CUS daily for 4 weeks and chronically treated with saline or fingolimod (see above) starting after 3 weeks of CUS. The first set of mice (Fig. 1A) was used for the assessment of depressive-like behavior in the forced swim test (FST) prior to the

onset of CUS, at the end of the third week of CUS, and then 30 min after the last administration of saline or fingolimod. The sucrose preference test (SPT) was performed at baseline and after 3 weeks of CUS, 1 h after the FST. Mice were killed 24 h after the last FST session for biochemical analysis.

The second set of mice (Fig. 4A) was used for a battery of behavioral tests including the elevated plus maze (EPM, performed prior to the onset of CUS and 12 h after the last administration of saline or fingolimod), the “single-day” Morris water maze (MWM) (performed 3 days after the end of treatments), and the social interaction test (performed 4 days after the MWM). At the end of the last behavioral session, all mice were treated with 5-bromo-2'-deoxyuridine (BrdU; Sigma Aldrich) and killed after 2 days for the assessment of hippocampal neurogenesis.

In the corticosterone model, mice were injected s.c. once daily for 21 days with either vehicle (1% Dimethyl sulfoxide (DMSO) in saline solution) or 20 mg kg⁻¹ of corticosterone. Corticosterone was initially dissolved in DMSO (100 mg mL⁻¹), and then diluted 1:100 into saline. The injection volume was 400 µL. Mice that had received corticosterone were treated i.p. for 4 additional weeks with saline or fingolimod (3 mg kg⁻¹). Mice that had received the vehicle alone, were exclusively treated with saline. The FST and SPT were carried out with the sequence described above at baseline, at 3 weeks (at the end of treatment with corticosterone or vehicle), and at 7 weeks (at the end of treatment with fingolimod or saline).

Chronic stress procedure

Mice were subjected to various unpredictable stressors for 28 days. For the first 21 days of stress, we used a modified version of the CUS protocol described by Koo et al. (2010). The stress protocol consisted of 1–3 h sessions in the morning and an overnight session (Table 1). The following stressors were delivered in the first 3 weeks: food deprivation for 12 h; 45° cage tilt for 12 h; wet bedding (250 mL of water in 750 mL of bedding) for 12 h; overnight illumination; 1-h restraint stress in a 15 × 5-cm cylinder; cage rotation for 1 h; different partner for 3 h; strobe light exposure overnight; overcrowding for 12 h; and light off for 3 h. During the last week of stress, the following items were intensified to avoid habituation or coping strategies: (i) 12 × 3 cm plastic tubes with tip cut off to allow breathing were used for restraint stress; (ii) wet bedding and cage rotation were combined for 3 h; and (iii) the light–dark cycle was reversed during the last weekend. In experiment #1, the CUS procedure was interrupted for 24 h after day 21. During this time, mice were subjected to FST, and then caged individually for 20 h for

the assessment of sucrose preference. There was no interruption of the CUS procedure experiment #2. Control mice were not exposed to stress and were maintained undisturbed in their home cages.

FST

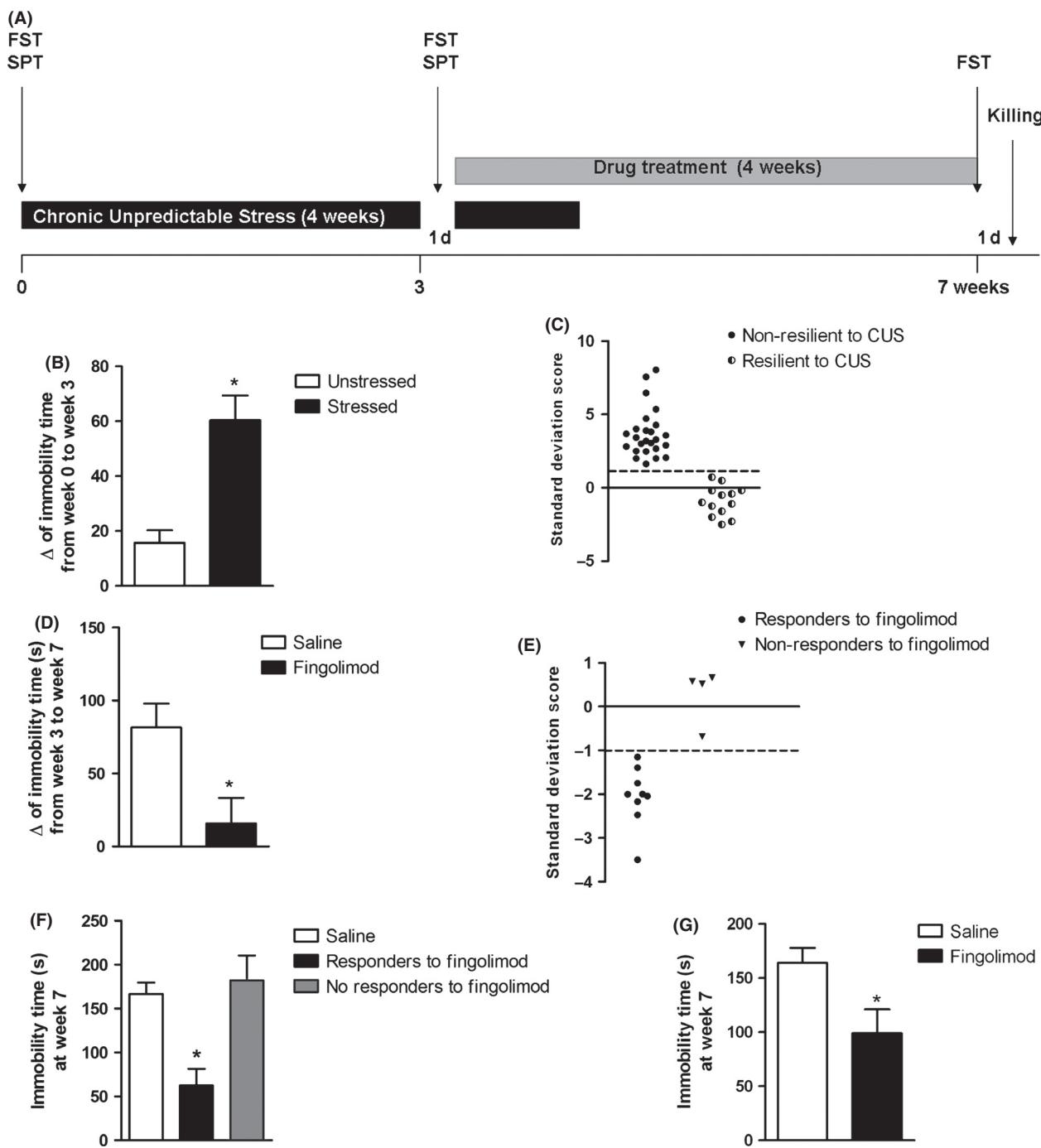
The FST (Porsolt et al. 2001) was performed in the morning (from 8 to 11 AM). Mice were allowed to adapt to the experimental room 1 h before testing. The third FST session (at the end of treatments) was performed 30 min after the injection of saline or fingolimod. Two mice were placed simultaneously in individual side-by-side Plexiglas cylinders (13 cm diameter × 24 cm high) containing 15 cm of water at 22–23°C, separated by an opaque screen. Mice were videotaped, and the immobility time was calculated in the last 4 min of the 6-min session. A mouse was judged immobile when it stopped all movements except those necessary to keep its head above the water.

SPT

The SPT was performed for 20 h starting at 12 PM (i.e., 1 h after the end of the FST). Mice were caged individually with free access to two different drinking bottles, the first containing water and the other filled with a 2% sucrose solution. The position of the two bottles was switched after 10 h from the beginning of the test, in order to avoid any side preference in drinking behavior. CUS mice had not been exposed to food or water deprivation for several days prior to the SPT (see Table 1). The preference for sucrose solution was assessed by weighing the bottles and calculated as a percentage of total fluid consumption.

EPM

The EPM was used as a test of anxiety (Rodgers and Dalvi 1997). The test was performed for the first time after 3 days of habituation to the experimenters. The maze, placed in a completely dark room, is a cross-shaped apparatus elevated 50 cm from the floor with two opposed open arms (5 × 30 cm), two closed arms (5 × 30 × 20 cm), and an open central zone (5 × 5 cm). Mice were put in the central zone with the head in the direction of one of the open arms and left free to move on the apparatus for 5 min. No training was performed, therefore, the animal's behavior was spontaneous and unconditioned. Measurements of the time spent and the number of entries in both open and closed arms were considered as measures of locomotor and exploratory activity. The performance of all animals was videotaped with a camera mounted above the maze and the videos were then ana-



lyzed with the EthovisionXT® software (Ethovision 8.5; Noldus Information Technology, Wageningen, The Netherlands).

MWM

We adopted a modified protocol of the MWM using a circular pool with a diameter of 100 cm and a height of

34 cm, filled with $20 \pm 1^\circ\text{C}$ water to a depth of 25 cm, with nonreflective interior surfaces, was placed in a completely dark room. The maze was divided into four equal quadrants and release points were assigned at quadrants NW, SW, and SE. A hidden circular platform (11 cm in diameter), was located in the center of the NE quadrant, submerged 1.5 cm beneath the surface of the water. A nontoxic paint was used to make the water white. Two

Figure 1. Antidepressant-like activity of Fingolimod in mice exposed to chronic unpredictable stress (CUS). The design of experiment #1 is shown in (A). Mice were exposed to CUS for 4 weeks, with 1 day of interval after the end of the third week for the analysis depressive-like behavior in the forced swim test (FST) and sucrose preference test (SPT). A four-week treatment with saline or Fingolimod (3 mg kg⁻¹, once daily) was started at the beginning of the 4th week of CUS. Mice were killed 1 day after the last FST. Cumulative data of the FST in all unstressed and CUS (stressed) mice ($n = 21$ and 38 , respectively) is shown in (B), where data (means + SEM) are expressed as the difference of immobility time (Δ) between values obtained at week 3 and time 0. * $P < 0.05$ (Student's t -test; $t_{(57)} = 3.53$) versus unstressed mice. Mice resilient ($n = 13$) and nonresilient ($n = 25$) to stress are shown in (C), where the cutoff value for resilience was considered <1 Standard Deviation Score (SDS) with respect to the mean value of immobility time in unstressed mice. SDS = $(x - \mu)/\sigma$, where x = the immobility time of each CUS mouse; μ and σ = mean and SD of the immobility time in unstressed mice. The overall effect of Fingolimod on the immobility time in the FST is shown in (D), where data (means + SEM) are expressed as the difference of immobility time (Δ) between values obtained at week 7 and week 3. * $P < 0.05$ (Student's t test; $t_{(20)} = 2.62$) versus mice treated with saline ($n = 9$ and 13 mice treated with saline and Fingolimod, respectively). Mice responsive and not responsive to the antidepressant-like activity of Fingolimod are shown in (E), where the cutoff for drug response was considered ≤ 1 SDS with respect to the mean value of immobility time in CUS mice treated with saline. Absolute values of immobility time in nonresilient CUS mice treated with saline ($n = 9$) or Fingolimod ($n = 13$, divided into nine responders and four nonresponders) are shown in (F), where values are means + SEM. One-way ANOVA + Tukey's t -test; $F_{(2,19)} = 12.86$; $P < 0.05$; post hoc analysis: (*) $P < 0.05$ versus all other values. In (G), data obtained in responders and nonresponders to Fingolimod were combined and compared to data obtained in mice treated with saline. * $P < 0.05$ (Student's t -test; $t_{19} = 2.141$).

Table 1. Chronic stress protocol.

Days of stress	Stressor	Duration	Modifications during week 4
1, 8, 11, 24	Food deprivation	Overnight	Food and water deprivation
1, 2, 9, 13, 20, 22, 25	Cage rotation	1 h	Cage rotation for 3 h
2, 7, 9, 13, 20, 26	Light on	Overnight	Inversion of light/dark cycle (day 26)
4, 7, 11, 15, 17, 26, 28	Light off	3 h	Inversion of light/dark cycle (day 26)
5, 8, 12, 19, 21, 23, 24	Different partner	3 h	In overcrowding
3, 14, 16, 19, 21, 23, 25	Cage tilt	Overnight	In overcrowding
3, 6, 10, 14, 16, 18, 27	Restraint	1 h	Restraint in 50-mL plastic tubes
6, 10, 17, 27	Strobe	Overnight	In overcrowding
5, 12, 15, 18, 28	Wet bedding	Overnight	In overcrowding

fixed, visual cues were present at various locations (NE and SW) around the maze. The task required mice to swim to the hidden platform guided by distal cues. Mice were subjected to three blocks of three trials each, with an interblock interval of at least 30 min, during which animals were returned to their home cage. Three different starting positions were equally distributed around the perimeter of the maze and randomized within each block. After mounting the platform, mice were allowed to remain there for 10 sec until the start of the next trial. The animals were given a maximum of 90 sec to find the platform; if they failed to find the platform in this time, they were placed by the experimenter on the platform and allowed to stay there for 10 sec. After completion of each block of trials, animals were returned to their home cage. One hour after the end of the three blocks, an extra 90-sec trial (probe trial) was performed. During probe trial, the platform was removed and the time spent by animals in each quadrant was recorded. A camera was mounted above the center of the maze and automated tracking was performed using the EthovisionXT® software (Ethovision 8.5, Noldus Information Technology). For all

the trials, both the time spent to find the platform and the total distance were measured. The time spent in each quadrant and in the circular zone of platform and near platform was recorded during the probe trial.

Social interaction test

The apparatus, placed in a totally dark room, consisted of a three-chamber plexiglass box with a nonreflective white floor and walls (outer: 60 × 40 × 22 cm; chambers: 20 × 40 × 22 cm). Rectangular openings (5 × 8 cm) were located on the inner dividing walls, so that mice were free to move across all the chambers of the box. Two cylindrical cages (internal diameter: 7 cm; height: 17 cm) with metal mesh walls and two white plastic caps were positioned in the distant corner of the outer chambers. Animals were habituated to the apparatus for 10 min, 2 days before the test. Testing consisted of three consecutive trials: habituation/acclimation to the environment, social interest (SI), and social recognition (SR). Habituation lasted for 5 min, while SI and SR lasted for 3 min; each step was delayed for 5 min, and during

intervals animals were put in their home cage. For each trial, mice were initially positioned in the central chamber and then were left free to explore the apparatus. During the SI trial, mice were exposed to a “familiar” conspecific placed in one of the two metal cages, while the other cage remained empty. During the SR trial, the “familiar” conspecific was left in the same cage, and in the empty one a “novel” conspecific was placed. The cage of the “familiar” conspecific was left in the same position during the SR trial. Conspecifics were habituated to stay in the metal cages inside the box for 5 min in the 2 days preceding the test day. Animal behavior in all trials was video-recorded and time spent in exploration of three chambers and of the metal cages was analyzed and calculated with the EthovisionXT® software (Ethovision 8.5, Noldus Information Technology).

Immunostaining and stereological counting of BrdU- or DCX-expressing cells in the dentate gyrus

For immunohistochemical analysis of cells expressing BrdU and doublecortin (DCX), brains were fixed in Carnoy's (ethanol: acetic acid: chloroform, 6:1:3), embedded in paraffin, and sectioned at 20 μm . Deparaffinized sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were treated with 10-mmol/L citrate buffer (pH 6.0), and heated in a microwave for 10 min for antigen retrieval. The slides were allowed to cool for 20 min in the same solution at room temperature and then washed in TBS (Tris-buffered saline). The sections were incubated overnight with mouse monoclonal anti-BrdU (1:10, Becton Dickinson, Franklin Lakes, NJ, USA) or goat polyclonal anti-DCX (1:20, Santa Cruz Biotechnology Inc., Dallas, Texas, U.S.A.) antibodies, and then for 1 h with secondary biotinylated anti-mouse or anti-rabbit antibodies (1:200; Vector Laboratories, Burlingame, CA). 3,3-Diaminobenzidine tetrachloride was used for detection (ABC Elite kit; Vector Laboratories). Control staining was performed without the primary antibodies.

The number of DCX⁺ neurons or BrdU⁺ cells in the dentate gyrus of the hippocampus was assessed by stereological technique and an optical fractionator using a Zeiss Axio Imager M1 microscope (Carl Zeiss Microscopy, Thomwood, NY, U.S.A.) equipped with a motorized stage and focus control system (Zeta axis), and with a digital video camera. The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Inc., Bethesda, MD) equipped with a Macro was used for the analysis of digital images. The Macro was obtained by Immagine and Computer, Bareggio, Italy and the characteristics of this Macro are published (King et al. 2002). The analysis was performed

on eight sections of 20 μm (for either BrdU or DCX staining), sampled every 200 μm on the rostro-caudal extension of the hippocampus, in which the dentate gyrus was identified and outlined at 2.5 \times magnification. DCX or BrdU-positive cells were counted at 100 \times magnification, with 1.3 as numerical aperture of the lens, as described (Gundersen and Jensen 1987). For stereological analysis, we used a grid of disectors with these characteristics: counting frame of 60 \times 60 μm ; grid size 150 \times 150 μm . The total number of DCX-positive cells per dentate gyrus was computed from the formula: $N = \Sigma(n) \times 1/\text{SSF} \times 1/\text{ASF} \times 1/\text{TSF}$, where n is the total number of cells counted on each disector; SSF (fraction of sections sampled) the number of regularly spaced sections used for counts divided by the total number of sections across the striatum (= 1/8); ASF (area sampling frequency) the disector area divided by the area between disectors (3600 μm^2 \times disector number/region area); and TSF (thickness sampling frequency) the disector thickness divided by the section thickness (19/20 μm).

Western blot analysis

Mice were killed by decapitation 24 h after the last behavioral session. The hippocampus and prefrontal cortex were dissected and stored at -80°C. Tissue was homogenized at 4°C in 0.1% Standard Deviation Score (SDS)-lysis buffer containing 1 mmol/L of a cocktail of protease inhibitors (Sigma, Milan, Italy), pH 7.4. Homogenates were centrifuged at 13,000g at 4°C for 20 min and the supernatant was used for protein determinations. About 30 μg of proteins were resuspended in SDS-bromophenol blue reducing buffer containing 40-mmol/L dithiothreitol and separated by electrophoresis on 12% SDS polyacrylamide gels, and later transferred to nitrocellulose membranes (Bio-Rad, Segrate, Milan, Italy). Transfer was performed at 4°C for 2 h in a buffer containing 25 mmol/L TRIS (Tris (hydroxymethyl) aminomethane), 192 mmol/L glycine, and 20% methanol, at 360 mA. Filters were blocked 10 min at 4°C in TBST= TBS Tween buffer containing 10% nonfat dry milk and then incubated with gentle shaking with the following primary antibodies: anti-BDNF (N-20) (rabbit polyclonal, 1:800, overnight at 4°C; Santa Cruz Biotechnology), anti-HDAC2 (mouse monoclonal, 1:5000, overnight at 4°C; Abcam, Cambridge, UK), anti-H3K14Ac (rabbit polyclonal, 1:1000, overnight, Upstate), and anti- β -Actin (mouse monoclonal, 0.5 $\mu\text{g mL}^{-1}$, 1 h at RT; Sigma-Aldrich). After three washes with TTBS buffer, blots were incubated for 1 h at room temperature with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (Sigma-Aldrich). Immunostaining was revealed by enhanced chemiluminescence (Amersham Biosciences,

Milan, Italy). Densitometric analysis was performed with Quantity One software (Bio-Rad).

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Monza MB, Italy) according to the manufacturer's protocol, and retrotranscribed into cDNA by using SuperScript III Reverse Transcriptase (Invitrogen). Real-time RT-PCR was performed on a Step One Plus Applied Biosystems. PCR was performed by using Power SYBR Green PCR Master Mix Kit (Applied Biosystems, Monza MB, Italy) according to the manufacturer's instructions. Thermal cycler conditions were as follows: 10 min at 95°C, 40 cycles of denaturation (30 sec at 95°C), and combined annealing/extension (1 min at 58°C). The following primers were used: Total *Bdnf*: forward 5'-CAG-GTCGAGAGGCTGACGA-3' and reverse 5'-CGCGTCCTATGGTTTCTCG-3'; *Hdac2*: forward 5'-GGGACAGGCTTGGTTGTTTC-3' and reverse 5'-GAGCATCAGCAATGGCAAGT-3'; *Gapdh*: forward 5'-CGTC CCGTAGACAAATGGT-3' and reverse 5'-TCAATGAAGGGTCGTTGAT -3'. The amount of mRNA was calculated from serially diluted standard curves simultaneously amplified with the samples and normalized with respect to *Gapdh* mRNA levels.

Statistical analysis

Statistical analysis was performed by Student's *t*-test (Figs. 1B, D, G, and 7B, D, E), one-way analysis of variance (ANOVA) + Tukey's *t*-test (Figs. 1F, 2G, 3A, and B), or two-way ANOVA + Fisher's LSD (Figs. 2A, C, E, F, 4C, 5A, B, and 6A, B).

Results

Effect of fingolimod treatment on CUS-induced depressive-like behaviour and depression-related biochemical changes in the hippocampus and prefrontal cortex

Antidepressant-like activity of fingolimod in mice exposed to CUS

The effect of CUS on depressive-like behavior was examined using the FST and SPT. The two tests were performed with 1 h of interval prior to the onset of CUS, and then after 3 weeks of CUS. At 3 weeks, CUS increased the immobility time in the FST in 66% of mice, whereas it reduced sucrose preference only in 30% of mice. There was only a partial overlapping between the

groups of responsive mice in the two tests. Hence, we only used the FST for the assessment of the antidepressant-like activity of fingolimod. Mice showing a reduced immobility time after 3 weeks of CUS were treated i.p. with saline or fingolimod (3 mg kg⁻¹) once a day for 28 days. Unstressed mice were also treated with saline or fingolimod for 4 weeks (Fig. 1A).

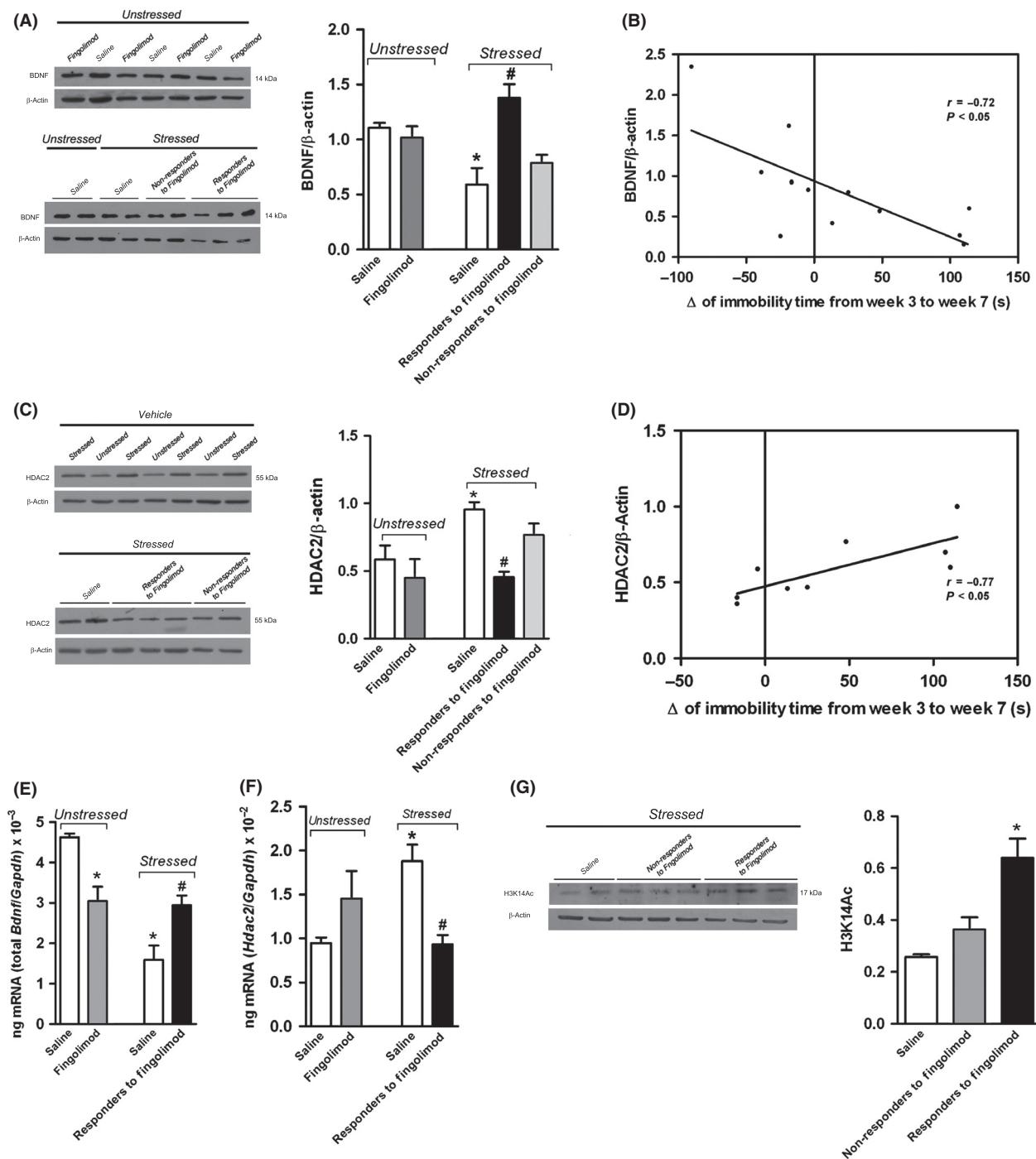
Three weeks of CUS significantly increased the immobility time as compared to baseline values (Fig. 1B). We arbitrarily considered CUS-exposed mice as "non-resilient" to stress if they showed at least + 1 SDS in the variation of the immobility time at 3 weeks, with respect to the mean variation in unstressed mice. Twenty-five of the 38 mice exposed to CUS were classified as "non-resilient" to stress and used for further analysis (Fig. 1C).

Treatment with saline for 4 weeks further increased the immobility time in stressed mice, perhaps because of the additional exposure to CUS during the first week of treatment. This increase was significantly reduced in mice treated with fingolimod (Fig. 1D). Although the overall effect of fingolimod was statistically significant, the drug was not effective in all CUS mice. At the end of treatment, nine of the 13 mice treated with fingolimod showed a reduction in the immobility time < -1 SDS with respect to the mean of saline-treated mice and were considered as "drug-responders." The four mice with a reduction in the immobility time < -1 SDS were classified as nonresponders (Fig. 1E). Absolute values are reported in Figure 1F, which shows a significant reduction in the immobility time only in the group of responder mice. The effect of fingolimod was still significant when data of responders and nonresponders were combined and compared to data obtained in mice treated with saline (Fig. 1G). Treatments with saline or fingolimod had no effect on the immobility time in unstressed mice (not shown).

CUS-induced changes in the expression of BDNF and HDAC2 in the hippocampus were corrected by fingolimod

As biochemical correlates with depressive-like behavior, we measured BDNF and HDAC2 mRNA and protein levels, and H3 histone acetylation in the hippocampus. BDNF and HDAC2 were also measured in the prefrontal cortex.

Stressed mice treated with saline showed a large reduction in hippocampal BDNF protein levels, as compared to unstressed mice treated with saline. Treatment with fingolimod reversed the effect of CUS on BDNF levels exclusively in mice that responded to the drug in the FST (Fig. 2A). Fingolimod did not change hippocampal BDNF levels in unstressed mice (Fig. 2A). Linear regression analysis showed a significant negative correlation between



BDNF levels and the immobility time in the FST ($r = -0.72$; $P < 0.05$) (Fig. 2B). Specular data were obtained with measurements of HDAC2 protein levels in the hippocampus. CUS caused a significant increase in HDAC2 levels, which was reversed by fingolimod only in “responder” mice. Fingolimod had no effect on HDAC2 levels in unstressed mice (Fig 2C). A positive correlation was found between HDAC2 levels and the immobility

time ($r = 0.77$; $P < 0.05$) (Fig. 2D). Data of BDNF and HDAC2 mRNA levels in CUS mice paralleled data of protein levels, with fingolimod reversing the effect of stress in responder mice. In unstressed mice, fingolimod caused a significant reduction in BDNF mRNA levels (Fig. 2E), although it did not change BDNF protein levels (Fig. 2A). Fingolimod had no effect on HDAC2 mRNA levels in unstressed mice (Fig. 2F). We also measured H3K14Ac in

Figure 2. Fingolimod treatment corrects biochemical changes caused by CUS in the mouse hippocampus. The effect of CUS and/or fingolimod on BDNF protein levels in the hippocampus is shown in (A). Densitometric values are means + SEM $n = 7$ and 6 for unstressed mice treated with saline and fingolimod, respectively; $n = 8$ and 13 for CUS (stressed) mice treated with saline and fingolimod, respectively. Stressed mice treated with fingolimod were subdivided into responders and nonresponders ($n = 9$ and 4, respectively). Two-way ANOVA + Fisher's LSD; stressed/unstressed: $F_{(1,29)} = 10.12$; drug treatment: $F_{(3,29)} = 9.52$; $P < 0.05$. Post hoc analysis: $P < 0.05$ versus unstressed mice treated with saline (*) or versus all other values except values in unstressed mice treated with saline (#). Linear regression analysis of BDNF levels and Δ values of immobility time between week 7 and week 3 in CUS mice treated with fingolimod ($n = 13$) is shown in (B). The effect of CUS and/or fingolimod on HDAC2 protein levels in the hippocampus is shown in (C). Densitometric values are means + SEM $n = 7$ and 4 for unstressed mice treated with saline and fingolimod, respectively; $n = 8$ and 9 for stressed mice treated with saline and fingolimod, respectively. Stressed mice treated with fingolimod were subdivided into responders and nonresponders ($n = 5$ and 4, respectively). Two-way ANOVA + Fisher's LSD; stressed/unstressed: $F_{(1,23)} = 11.99$, $P < 0.05$; drug treatment: $F_{(3,23)} = 6.39$, $P < 0.05$. Post hoc analysis: $P < 0.05$ versus unstressed mice treated with saline (*) or versus stressed mice treated with saline and nonresponders to fingolimod (#). Linear regression analysis of HDAC2 levels and Δ values of immobility time between week 7 and week 3 in CUS mice treated with fingolimod ($n = 13$) is shown in (D). The effect of CUS and/or fingolimod on BDNF and HDAC2 mRNA levels in the hippocampus is shown in (E) and (F), respectively. Values are means + SEM of 3–4 determinations in (E) and 2–5 determinations in (F). Two-way ANOVA + Fisher's LSD; BDNF mRNA levels, stressed/unstressed: $F_{(1,11)} = 30.063$, $P < 0.05$; drug treatment: $F_{(1,11)} = 0.805$, $P > 0.05$; stressed/unstressed \times drug treatment: $F_{(1,11)} = 25.849$, $P < 0.05$; HDAC2 mRNA levels, stressed/unstressed: $F_{(1,10)} = 3.141$, $P > 0.05$; drug treatment: $F_{(1,10)} = 3.529$, $P > 0.05$; stressed/unstressed \times drug treatment: $F_{(1,10)} = 19.368$, $P < 0.05$. Post-hoc analysis: $P < 0.05$ versus the respective unstressed mice treated with saline (*) or the respective stressed mice treated with saline (#). H3K14 Ac levels in the hippocampus of CUS mice treated with saline or fingolimod (divided into responders and nonresponders) are shown in (G), where data are means + SEM of 2–3 determinations (SD is reported for the saline group in which $n = 2$). One-way ANOVA + Tukey's *t*-test; $F_{(2,5)} = 11.09$; $P < 0.05$. Post hoc analysis: * $P < 0.05$ versus all other values.

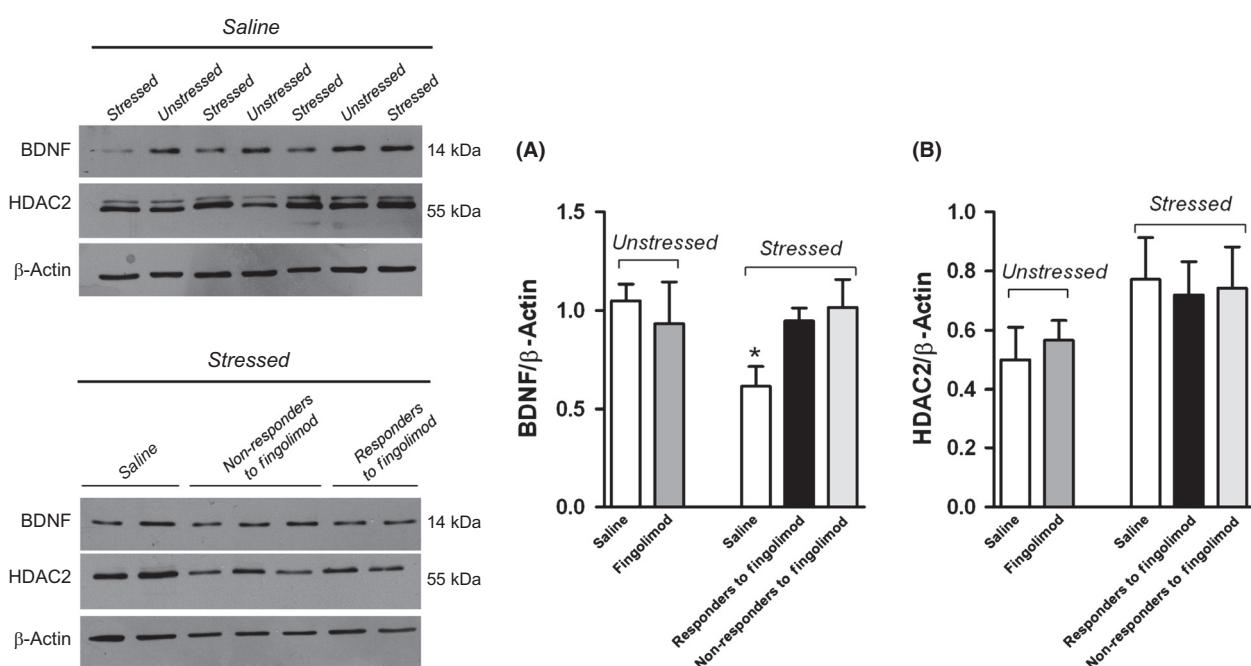


Figure 3. Fingolimod treatment did not cause significant changes in cortical BDNF and HDAC2 levels in stressed mice classified as "drug responders." BDNF and HDAC2 levels in the prefrontal cortex of unstressed and CUS (stressed) mice treated with saline or fingolimod are shown in (A) and (B), respectively. Values are means + SEM. In (A), $n = 9$ and 4 in unstressed mice treated with saline and fingolimod, respectively; $n = 8$ in mice treated with saline and fingolimod (four responders and 4 nonresponders). In (B), $n = 7$ and 3 in unstressed mice treated with saline and fingolimod, respectively; $n = 8$ and 11 in mice treated with saline and fingolimod, respectively (seven responders and four nonresponders to fingolimod). Two-way ANOVA + Fisher's LSD; BDNF levels, stress/no stress: $F_{(1,24)} = 9.96$, $P < 0.05$; drug treatment: $F_{(1,24)} = 2.445$, $P > 0.05$; HDAC2 levels, stress/no stress: $F_{(1,24)} = 2.77$, $P > 0.05$; drug treatment: $F_{(3,24)} = 0.06$, $P > 0.05$. Post hoc analysis: * $P < 0.05$ versus the respective values obtained in unstressed mice treated with saline.

the hippocampus of CUS mice. Fingolimod treatment enhanced H3K14Ac levels only in "responder" mice (Fig. 2G).

In the prefrontal cortex, exposure to CUS significantly reduced BDNF protein levels but did not cause significant changes in HDAC2 protein levels. Mice treated with

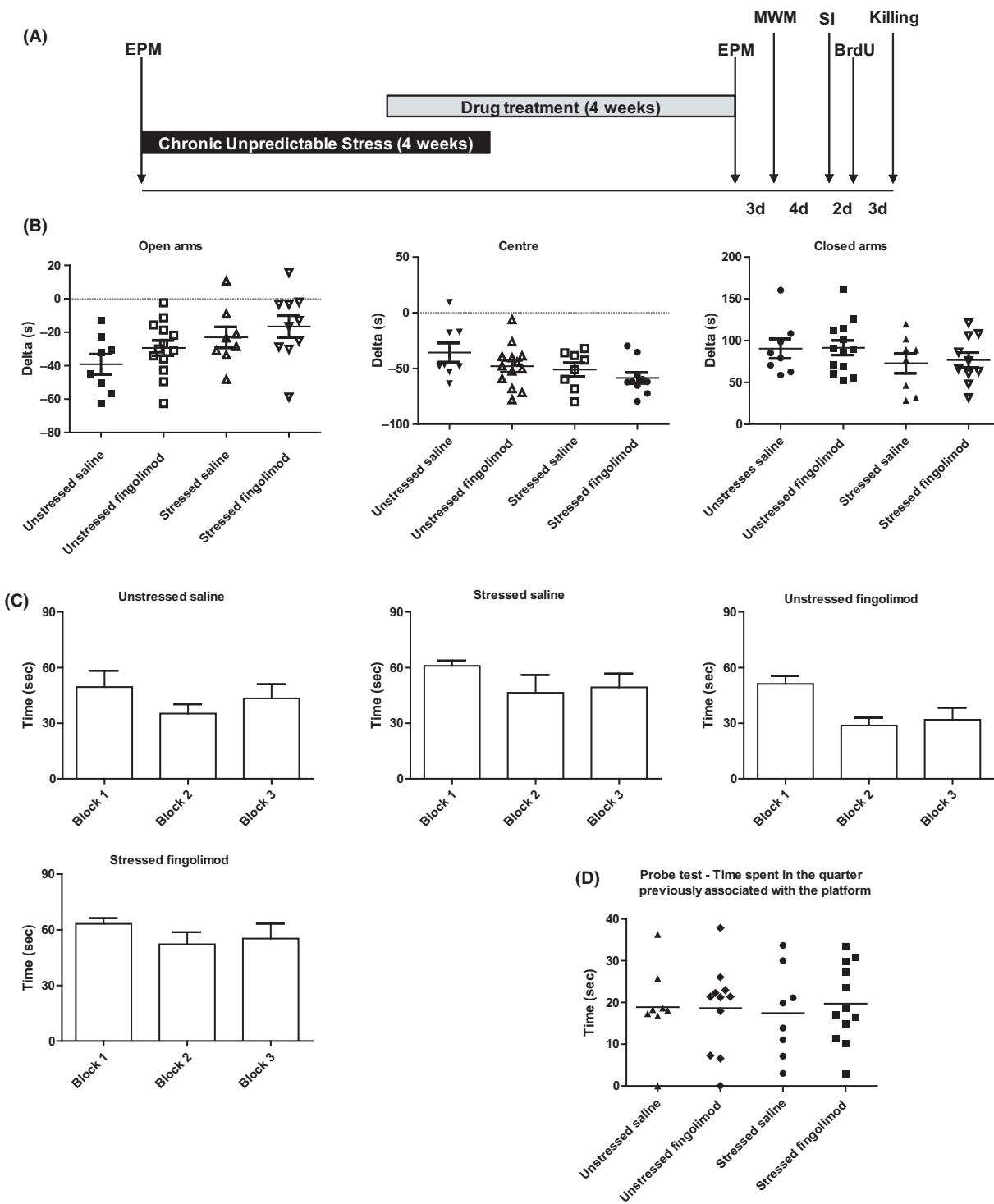


Figure 4. Fingolimod treatment had no effect on anxiety-like behavior in the EPM or spatial learning in the 1 day Morris water maze. The design of experiment #2 is shown in (A). Single values at the EPM are shown in (B). Values (means + SEM) of the three blocks of trials in the water maze are shown in (C). $n = 8$ and 11 for unstressed mice treated with saline and fingolimod, respectively; $n = 8$ and 12 for stressed mice treated with saline and fingolimod, respectively. Two-way ANOVA for repeated measures showed a significant difference among the blocks ($F_{2,36} = 7.54$; $P < 0.05$), a significant effect of CUS ($F_{1,36} = 13.44$; $P < 0.05$), and no effect of treatment ($F_{1,36} = 0.048$; $P > 0.05$) or CUS \times treatment ($F_{1,36} = 2.14$; $P > 0.05$). Single values of the probe test (the time spent in the quarter of the maze previously associated with the platform) are shown in (D). EPM, elevated plus maze; MWM, morris water maze; SI, social interaction test.

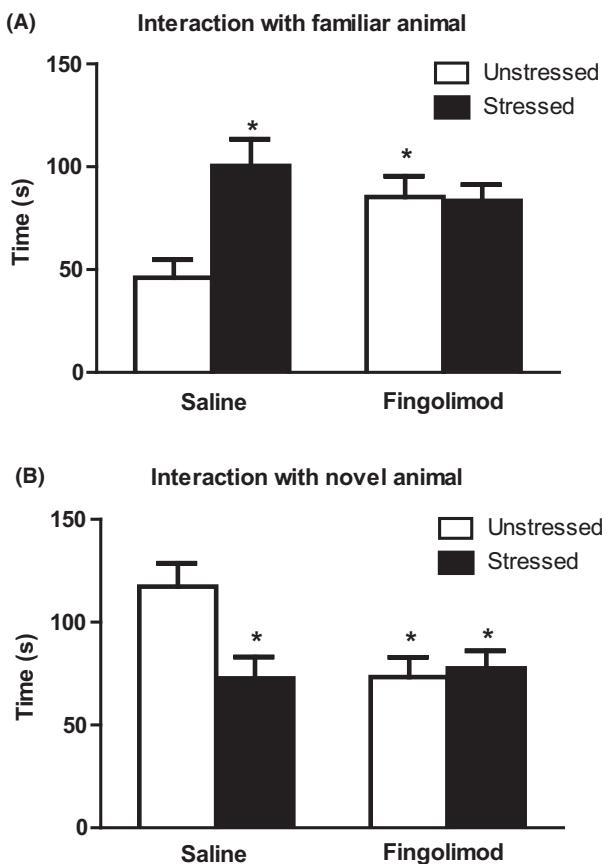


Figure 5. Fingolimod treatment mimicked and occluded the action of CUS in the social interaction test. Values of the recognition phase of test relative to the interaction with the familiar and the novel conspecific are shown in (A) and (B), respectively. Values are means + SEM $n = 7$ and 11 for unstressed mice treated with saline or fingolimod, respectively; $n = 7$ and 12 for CUS (stressed) mice treated with saline and fingolimod, respectively. Two-way ANOVA + Fisher's LSD; time spent with the familiar conspecific, stressed/unstressed: $F_{3,33} = 6.63$, $P < 0.05$; drug treatment: $F_{3,33} = 1.205$, $P > 0.05$; stressed/unstressed \times drug treatment, $F_{3,33} = 7.64$, $P < 0.05$; time spent with the novel conspecific, stressed/unstressed: $F_{3,33} = 3.96$, $P > 0.05$; drug treatment: $F_{3,33} = 3.71$, $P > 0.05$; stressed/unstressed \times drug treatment, $F_{3,33} = 5.82$, $P < 0.05$. Post hoc analysis: * $P < 0.05$ versus the respective values obtained in unstressed mice treated with saline.

fingolimod showed a trend to an increase in BDNF levels, with no difference between responders and nonresponders (Fig. 3A). The drug had no effect on cortical HDAC2 levels (Fig. 3B).

Effect of CUS and fingolimod treatment on EPM, spatial learning, social interaction, and hippocampal neurogenesis

The second set of mice subjected to CUS and treated as above underwent behavioral analysis in the EPM, social interaction test, and 1 day MWM test, and were also used

for the assessment of neurogenesis in the hippocampal dentate gyrus (see Fig. 4A).

Fingolimod had no effect on anxiety-like behaviour in the EPM or spatial learning in the 1 day water maze

The EPM test was performed twice, prior to the onset of CUS and 24 h after the last injection of saline or fingolimod. We found no effects of CUS or fingolimod treatment in the EPM test, as reflected by the time spent by each mouse in the open and closed arms of the EPM (Fig. 4B). Spatial learning was assessed by 1 day MWM performed 3 days following the last EPM test. Mice were subjected to three blocks of three trials separated by a 30-min interval. Data from the three blocks of trials (Fig. 4C) and the probe test (Fig. 4D) showed no effect of fingolimod on spatial learning in both unstressed and CUS mice.

Fingolimod treatment mimicked and occluded the effect of CUS on social interaction

The social interaction test can be used as a test of anxiety-like behavior from an ethological perspective (File and Hyde 1978). Data from the SR phase of the test are shown in Fig. 5. Both CUS and fingolimod increased the time spent in proximity of the familiar conspecific (Fig. 5A), and reduced the time spent in proximity of the novel conspecific (Fig. 5B). CUS and fingolimod treatment were mutually occlusive in the social interaction test (Fig. 5A and B).

Fingolimod treatment reversed the reduction of adult hippocampal neurogenesis induced by CUS

At the end of the last behavioral session all mice were treated with BrdU and killed after 2 days (corresponding to day 12 after the end of treatments with saline or fingolimod) for the assessment of hippocampal neurogenesis. Exposure to CUS reduced the absolute number of proliferating progenitors in the dentate gyrus, as assessed by stereological counting of BrdU⁺ cells. The effect of CUS was reversed by fingolimod (Fig. 6A). Similar data were obtained by measuring the number of cells expressing DCX, a microtubule-associated protein specifically found in neuronal precursor cells and immature neurons (von Bohlen and Halbach 2011) (Fig. 6B).

Effect of fingolimod on corticosterone-induced depressive-like behaviour

After 21 days of treatment with corticosterone (or vehicle), the depressive-like behavior of mice was assessed

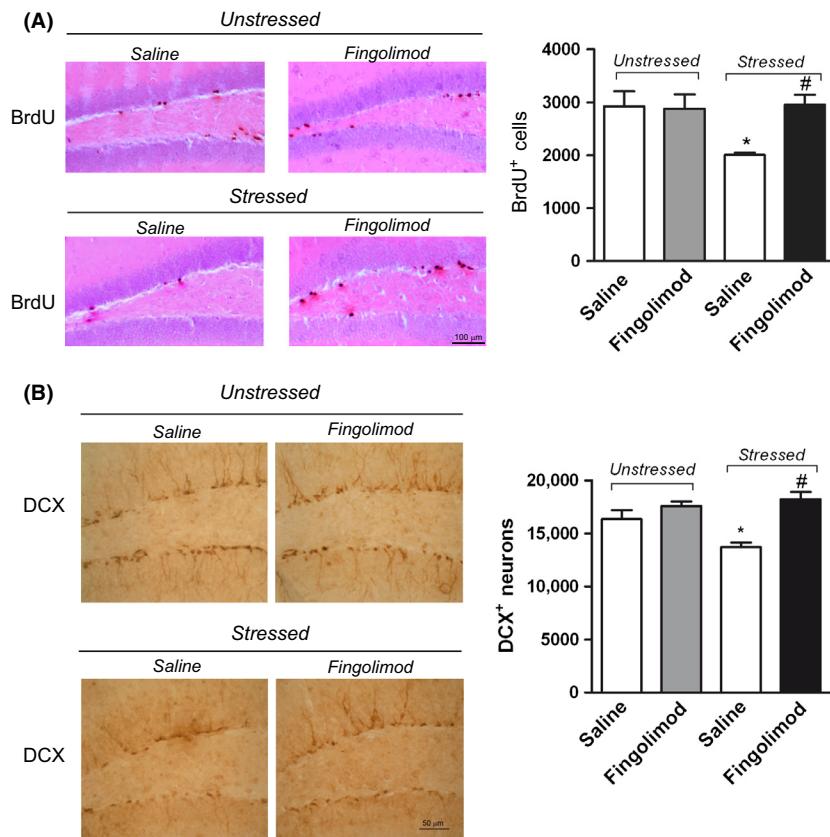


Figure 6. Fingolimod treatment enhances adult neurogenesis in the hippocampal dentate gyrus of mice exposed to CUS. Stereological counts of BrdU^+ or DCX^+ cells in the dentate gyrus of unstressed and CUS (stressed) mice treated with saline or fingolimod are reported in (A) and (B), respectively. Representative images of BrdU or DCX staining are also shown. Values are means + SEM of 5 determinations per group in (A) and 4 determinations per group in (B). Two-way ANOVA + Fisher' LSD; BrdU^+ cells, stressed/unstressed: $F_{(3,16)} = 3.829$, $P > 0.05$; drug treatment: $F_{(3,16)} = 4.366$, $P > 0.05$; stressed/unstressed \times drug treatment: $F_{(3,16)} = 5.273$, $P < 0.05$; DCX^+ cells, stressed/unstressed: $F_{(3,12)} = 2.483$, $P > 0.05$; drug treatment: $F_{(3,12)} = 20.898$, $P < 0.05$; stressed/unstressed \times drug treatment: $F_{(3,12)} = 6.768$, $P < 0.05$. Post hoc analysis: $P < 0.05$ versus the respective groups of unstressed mice treated with saline (*), or versus the respective groups of stressed mice treated with saline (#).

using the FST and the SPT, as described above. Compared to baseline values, corticosterone treatment increased the time spent in immobility in the FST (Fig. 7B) in 77% of mice (17 out of 22). We arbitrarily considered as “Responders to corticosterone” mice that showed at least +0.20 SDS in the variation of immobility time at 3 weeks, with respect to the mean variation in vehicle-treated mice (Fig. 7C).

Responders to corticosterone were then treated with fingolimod (3 mg kg^{-1}) or saline for 4 weeks. Fingolimod induced a significant reduction in the time spent in immobility, with respect to values obtained at 3 weeks (Fig. 7D). Fingolimod also significantly reduced the immobility time when compared to saline (Fig. 7E).

Treatment with corticosterone and fingolimod had no appreciable effects on the hedonic component of depressive-like behavior, as assessed with the SPT (data not shown).

Discussion

Our data show an antidepressant-like activity of fingolimod in mice exposed to CUS and in mice chronically treated with corticosterone. Stress-based models of depression, including the CUS model, have face validity and are highly responsive to antidepressant medication (Nestler and Hyman 2010; Krishnan and Nestler 2011). The corticosterone model mimics the dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis associated with major depression, and recapitulates some of the hallmark symptoms of depression (reviewed by Gourley and Taylor 2009). As opposed to the CUS model, the corticosterone model, under our experimental conditions, is refractory to classical antidepressants (Iijima et al. 2010; Ago et al. 2013). Fingolimod treatment was effective in both models in relieving depressive-like behavior in the FST. The two models,

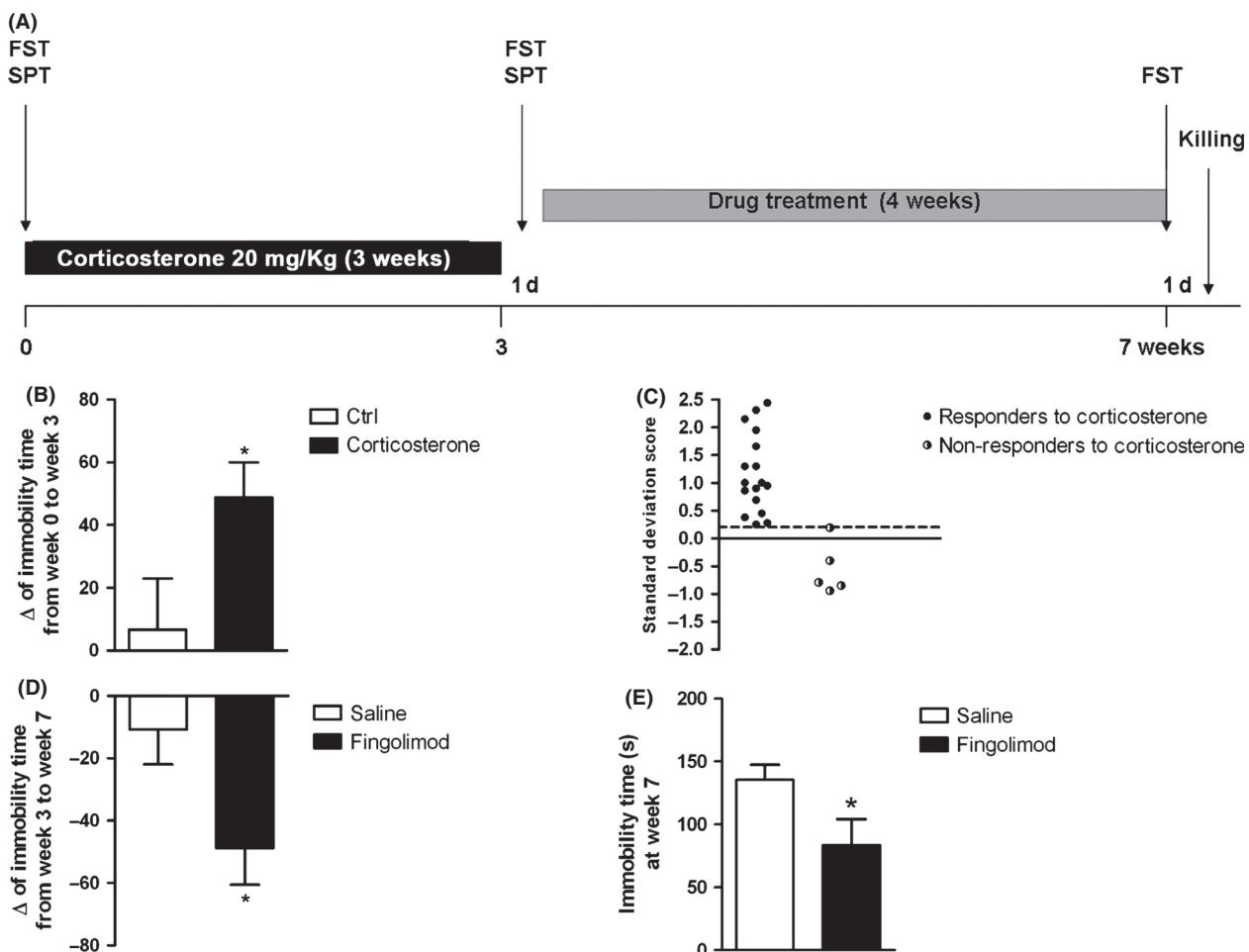


Figure 7. Antidepressant-like activity of fingolimod in mice chronically treated with corticosterone. The design of the experiment is shown in (A). Mice were treated systemically with corticosterone (20 mg kg^{-1} , s.c.) or vehicle (Ctrl) for 3 weeks, tested for depressive-like behavior in the forced swim test (FST) and sucrose preference test (SPT), and then treated i.p. once daily for 4 weeks with either saline (both groups) or fingolimod (3 mg kg^{-1} , only the corticosterone group). All data of the FST in Ctrl mice and in mice treated with corticosterone ($n = 11$ and 22, respectively) are shown in B, where data (means \pm SEM) are expressed as the difference of immobility time (Δ) between values obtained at week 3 and time 0. $*P < 0.05$ (Student's t -test; $t_{(31)} = 2.14$) versus unstressed mice. Mice responders ($n = 17$) and nonresponders ($n = 6$) to corticosterone are shown in (C), where the cutoff value for resilience was considered <0.20 Standard Deviation Score (SDS) with respect to the mean value of immobility time in control mice treated with vehicle. The effect of fingolimod on the immobility time in the FST is shown in (D), where data (means \pm SEM) are expressed as the difference of immobility time (Δ) between values obtained at week 7 and week 3. $*P < 0.05$ (Student's t -test; $t_{(15)} = 2.33$) versus mice treated with saline ($n = 7$ in both groups). Absolute values of immobility time (means \pm SEM) in mice treated with saline or fingolimod are shown in (E). $*P = 0.05$ (Student's t test; $t_{15} = 2.09$) versus saline.

however, did not allow the examination of the effect fingolimod on anhedonia. Mice treated with corticosterone for 3 weeks did not show the expected reduction in sucrose preference (Gourley and Taylor 2009), and only a small percentage of mice exposed to CUS showed anhedonia in the SPT. This was unexpected because inhibition of sucrose preference has been reported in C57/BL mice exposed to unpredictable chronic mild stress (Pothion et al. 2004; Cuccuruzzu et al. 2013; but see also Farley et al. 2012 for contrasting results). Further studies with other animal models

are required for the evaluation of the activity of fingolimod on anhedonic behavior.

A 4 week treatment with fingolimod in “non-resilient” CUS mice caused antidepressant-like effect in the FST. However, about 30% of CUS mice did not respond to fingolimod for unknown reasons. Interestingly, fingolimod corrected the abnormalities in BDNF levels, HDAC2 levels, and H3K14 acetylation in the hippocampus only in those mice that were classified as “responders” on the basis of behavioral data. This correlation was not found in the prefrontal cortex of CUS mice, where fingolimod

caused a trend to an increase in BDNF levels in *both* responders and nonresponders, and had no effect on HDAC2 levels. These findings suggest that fingolimod acts primarily in the hippocampus through an epigenetic mechanism that causes an enhanced production of BDNF, resulting in an antidepressant-like effect (see Introduction and References therein).

To the best of our knowledge, our data offer the first demonstration that hippocampal HDAC2 levels are increased in a model of chronic stress. This contrasts with the reduced HDAC2 mRNA levels found in the hippocampus of mice exposed to restraint stress for 14 days (Han et al. 2014). Changes in hippocampal HDAC2 levels found in response to CUS and fingolimod were complementary to changes in BDNF levels, in line with the evidence that HDAC2 epigenetically downregulates BDNF expression (Gräff et al. 2012). The ability of fingolimod to enhance H3K14Ac levels in CUS mice is fully consistent with its putative antidepressant activity because chronic social defeat stress – another validated stress-based model of depression – causes a persistent reduction in hippocampal H3K14Ac levels, which is reversed by imipramine treatment (Covington et al. 2011). Interestingly, acetylation of another H3 lysine residue (H3K9) is enhanced by a 3-day treatment with fingolimod in *severe combined immunodeficiency disorder* (SCID) mutant mice subjected to a contextual fear extinction test (Hait et al. 2014), suggesting that the drug may activate different epigenetic mechanisms depending on the context, length of treatment, and strain of mice.

Another finding that supports the antidepressant-like activity of fingolimod was the enhancing effect of the drug on hippocampal neurogenesis in CUS mice. Adult hippocampal neurogenesis is negatively regulated by chronic stress, and an increased neurogenesis contributes to some (but not all) “therapeutic” effects of antidepressant drugs (Santarelli et al. 2003; David et al. 2009; Hsieh and Eisch 2010; Hanson et al. 2011). Because of the complex design of experiment #2, hippocampal neurogenesis was assessed 12 days after the end of drug treatment. This suggests that fingolimod produces long-lasting effects (at least on hippocampal neurogenesis), which might depend either on the long half-life of the drug (>60 h) or to the enduring consequences of histone acetylation (see above).

In the same groups of mice used for the assessment of neurogenesis, we examined the effect of CUS and fingolimod on EPM, one-day water maze, and social interaction. Under our conditions, fingolimod had no effect on anxiety-like behavior in the EPM and spatial learning in the water maze. It should be highlighted that, under our experimental conditions, CUS did no cause changes in anxiety-like behavior or spatial learning. Thus, our data do not exclude an action of fingolimod on anxiety and

cognitive dysfunction associated with depression. In contrast, fingolimod mimicked and occluded the action of CUS in reducing the time spent in proximity of a novel animal in the recognition phase of the social interaction test. This particular effect of fingolimod might reflect an increased level of anxiety caused by the novel animal (File and Hyde 1978), but might also be secondary to an increased affiliative behavior toward the familiar animal. Hait et al. (2014) have shown that a 3-day treatment with fingolimod enhances the extinction of fear memory in SCID mutant mice. SCID mice represent an elegant model for the study of the central action of fingolimod without the influence of peripheral immune suppression, but are not an optimal model for the study of depression- and anxiety-like behavior because immune deficiency has a profound impact on the activity of the HPA axis. All together, these findings suggest that the effect of fingolimod on anxiety-like behavior is complex and requires further investigation.

Fingolimod is transformed inside the cell into fingolimod-P, which may interact with membrane S1PRs *via* an “inside-out” mechanism (Brinkmann et al. 2010), or, alternatively, inhibit class-I HDACs in the cell nucleus (Hait et al. 2014). Both mechanisms might contribute to the antidepressant-like activity of fingolimod and the associated increase in hippocampal BDNF levels and neurogenesis. The use of subtype-selective S1PR antagonists or conditional null mice lacking S1P1Rs in specific CNS cell lineages (Choi et al. 2011) is needed to examine the role of S1PRs in the antidepressant effect of fingolimod. At least under our conditions, fingolimod had no effect on depressive-like behavior, BDNF protein levels, HDAC2 mRNA and protein levels, and adult neurogenesis in unstressed control mice. A “disease-dependent” effect is also reported in a mouse model of Alzheimer’s disease, in which peripheral administration of fingolimod for two weeks reversed the reduction in hippocampal BDNF levels caused by i.c.v. infusion of full-length β -amyloid peptide, with no effect on BDNF levels in control mice (Fukumoto et al. 2014). In their seminal manuscript, Deogracias et al. (2012) have shown that a single injection of fingolimod enhances hippocampal BDNF mRNA and protein levels in *normal* mice. In our unstressed control mice, chronic treatment with fingolimod caused no changes in BDNF protein levels and even a significant reduction in BDNF mRNA levels in the hippocampus. Perhaps tolerance develops to the BDNF-enhancing effect of fingolimod after repeated injections of the drug unless BDNF levels are pathologically reduced, as occurs in CUS mice (present data), in mice injected with β -amyloid peptide (Fukumoto et al. 2014), or in mutant mice modeling Rett’s syndrome (Deogracias et al. 2012).

In conclusion, our findings raise the interesting possibility that fingolimod relieves depressive symptoms in MS patients independently of its established disease-modifying activity (Aktas et al. 2010; Brinkmann et al. 2010; Peltier and Hafler 2012; Comi 2013; di Nuzzo et al. 2014). Post hoc data from the phase 4 EPOC trial (ClinicalTrials.gov, identifier NCT01216072) presented as a poster at the 29th Congress of the European Committee for Treatment and Research in MS (Hunter et al. 2013) show that a larger proportion of MS patients who were depressed at baseline were no longer depressed 6 months after switching the therapy from IFN- β or glatiramer acetate (GA) into fingolimod with respect to patients who switched from IFN- β into GA or vice versa (50.5% vs. 25.3%). This encourages the design of controlled clinical trials in which the antidepressant activity of fingolimod is compared to the activity of drugs endowed with high therapeutic efficacy in MS patients, such as natalizumab.

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Disclosure

None declared.

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