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RESEARCH PAPER

Acute desipramine restores presynaptic cortical defects in murine experimental autoimmune encephalomyelitis by suppressing central CCL5 overproduction

Silvia Di Prisco[1]*, Elisa Merega[1]*, Massimiliano Lanfranco[2], Simona Casazza[3], Antonio Uccelli[3,4] and Anna Pittaluga[1,4]

[1]*Department of Pharmacy, DiFAR, Pharmacology and Toxicology Section, Genoa, Italy,* [2]*Drug Discovery and Development, Italian Institute of Technology, Genoa, Italy,* [3]*Department of Neurosciences, Ophthalmology and Genetics, University of Genoa, Genoa, Italy, and* [4]*Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy*

Correspondence
Professor Anna Pittaluga, Department of Pharmacy, Pharmacy and Toxicology Section, Viale Cembrano 4, Genova 16148, Italy. E-mail: pittalug@pharmatox.unige.it

*Equally contributed.

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BACKGROUND AND PURPOSE

Altered glutamate exocytosis and cAMP production in cortical terminals of experimental autoimmune encephalomyelitis (EAE) mice occur at the early stage of disease (13 days post-immunization, d.p.i.). Neuronal defects were paralleled by overexpression of the central chemokine CCL5 (also known as RANTES), suggesting it has a role in presynaptic impairments. We propose that drugs able to restore CCL5 content to physiological levels could also restore presynaptic defects. Because of its efficacy in controlling CCL5 overexpression, desipramine (DMI) appeared to be a suitable candidate to test our hypothesis.

EXPERIMENTAL APPROACH

Control and EAE mice at 13 d.p.i. were acutely or chronically administered DMI and monitored for behaviour and clinical scores. Noradrenaline and glutamate release, cAMP, CCL5 and TNF-α production were quantified in cortical synaptosomes and homogenates. Peripheral cytokine production was also determined.

KEY RESULTS

Noradrenaline exocytosis and α_2-adrenoceptor-mediated activity were unmodified in EAE mice at 13 d.p.i. when compared with control. Acute, but not chronic, DMI reduced CCL5 levels in cortical homogenates of EAE mice at 13 d.p.i., but did not affect peripheral IL-17 and TNF-α contents or CCL5 plasma levels. Acute DMI caused a long-lasting restoration of glutamate exocytosis, restored endogenous cAMP production and impeded the shift from inhibition to facilitation of the CCL5-mediated control of glutamate exocytosis. Finally, DMI ameliorated anxiety-related behaviour but not motor activity or severity of clinical signs.

CONCLUSIONS

We propose DMI as an add-on therapy to normalize neuropsychiatric symptoms in multiple sclerosis patients at the early stage of the disease.

Abbreviations

[^{3}H]-D-Asp, [^{3}H]-D-aspartate; CCL5, RANTES regulated upon activation normal T-cell expressed and secreted; CCR, chemokine receptor; d.p.i., days post immunization; DMI, desipramine; EAE, experimental autoimmune encephalomyelitis; GBR12909, 1-(2-(bis-(4-fluorophenyl)methoxy)ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride; Glu, glutamate; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NRI, noradrenaline re-uptake inhibitor

Introduction

Multiple sclerosis (MS) is a demyelinating, progressively degenerating disorder of the CNS. Although autoimmune mechanisms are known to play a major role, previous studies have suggested that impaired central neurotransmission (Groom *et al.*, 2003; Centonze *et al.*, 2009) is equally important in determining disease progression. In particular, classic neurotransmitters including glutamate (Glu) and NA were proposed to have a role in the etiopathogenesis of MS as well as of experimental autoimmune encephalomyelitis (EAE), the most used animal model of MS (Schwartz *et al.*, 2003; Carnevale *et al.*, 2007; Musgrave *et al.*, 2011; Polak *et al.*, 2011; 2012).

Recently, we carried out a comparative analysis of presynaptic defects in glutamatergic nerve terminals in two different CNS regions (i.e. the cortex and the spinal cord, Di Prisco *et al.*, 2013) of EAE mice. Our first conclusion was that both early and acute impairments of Glu transmission take place in these CNS regions. In particular, early presynaptic defects were observed in the cortex at 13 days post-immunization (d.p.i.), while Glu impairments in the spinal cord only became detectable at 21–30 d.p.i. Changes in Glu exocytosis were eventually paralleled by peripheral and central overexpression of the chemokine regulated upon activation normal T-cell expressed and secreted (RANTES, immunological designation CCL5), a pro-inflammatory chemokine whose pathological overproduction is a hallmark of disease progression (Sørensen *et al.*, 1999). Notably, in these CNS regions, impaired releasing efficacy in glutamatergic terminals was reminiscent of the presynaptic *in vitro* effects CCL5 exerts on Glu release in healthy conditions (Musante *et al.*, 2008; Di Prisco *et al.*, 2012), leading us to propose a strict correlation between presynaptic defects and the pathologically-relevant concentration of the endogenous chemokine in the synaptic cleft. If this is the case, compounds that could restore CCL5 content to physiological levels should also be expected to normalize Glu transmission. To test this hypothesis, we focused on the antidepressant desipramine (DMI), because of its ability to reduce endogenous CCL5 production triggered by systemic inflammatory stimuli (Reynolds *et al.*, 2005; O'Sullivan *et al.*, 2009; 2010). As a first approach, we investigated the effect of both acute and chronic DMI treatments in the cortex of EAE mice during the early stage of disease (13 d.p.i.). The striking results were that acute, but not chronic, DMI restored early presynaptic defects (i.e. Glu release and cAMP production) as well as CCL5 overexpression in this brain region, while the peripheral productions of TNF-α, IL-17 and CCL5 were unmodified. Based on these observations, we propose that acute DMI administration in the early phase of disease could represent an add-on therapy to restore the physiological cross-talk between nervous and immune systems and to normalize early neuropsychiatric symptoms in MS patients.

Methods

Animals and EAE induction

Animals were housed in standardized conditions at constant temperature (22°C) and relative humidity (50%) under a regular light/dark schedule (lights on, 07 h to 19 h) in standard cages (8 animals per cage), with food and water available *ad libitum*. Female mice (C57BL/6; 18–20 g, 6–8 weeks) were injected, s.c., with incomplete Freund's adjuvant containing 4 mg mL^{-1} *Mycobacterium tuberculosis* (strain H37Ra) and 200 μg of the myelin oligodendrocyte glycoprotein, sequence 35–55 ($MOG_{35–55}$). Immunization with $MOG_{35–55}$ was followed by i.p. administration of 400 ng pertussis toxin on day 0 and after 48 h. Clinical scores (Zappia *et al.*, 2005) were recorded before mice were killed. Control mice received the same treatment in the absence of the $MOG_{35–55}$ peptide. All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results. At 13 d.p.i., EAE mice were killed by decapitation, the cortices were rapidly removed and purified isolated nerve endings (synaptosomes) prepared within minutes. When indicated, EAE mice were killed at 21 d.p.i. All the experimental procedures described here were in accordance with the European legislation (European Communities Council Directive of 22 September 2010, no. 2010/63/EU), with the Italian legislation (L.D. no. 116 /1992), with the ARRIVE Guidelines and they were approved by the Italian Ministry of Health (protocol number no. 50/2011-B). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Drug treatments

Female C57BL/6 mice (18 mice for each set of experiments) were randomly assigned to the following groups: control mice, EAE mice, DMI-treated control mice, DMI-treated EAE mice. After a 3-day trial to determine the amount of water consumed by each group of mice, mice were treated with DMI (10 mg kg^{-1}) dissolved in the drinking water. In acute treatments, animals were administered drug for 24 h, in chronic treatments mice were administered DMI for 14 days, starting from the day of mouse immunization. When indicated, control DMI-treated mice were acutely injected 30 min before animals were killed with yohimbine (0.5 mg kg^{-1} i.p.; dissolved in 0.9% NaCl).

Rotarod

Motor performance was monitored with a Rotarod apparatus (accelerating 5-lane model; Ugo Basile, Varese, Italy). Mice were trained for 2 consecutive days before EAE induction. The latency to fall off a rotating rod was measured at a fixed speed of 16 r.p.m. for a maximum of 300 s. The motor performance of each mouse was analysed three times, from which the average time a mouse remained on the rotating beam was calculated.

Light dark box

Animals were placed in the centre of the light zone of the light dark box consisting of a lighted and a dark compartment (each comprising 35 cm × 30 cm × 21 cm) and were allowed to explore the box for 10 min. The total time spent in the lighted compartment and the number of transitions were analysed.

Open field test

Spontaneous locomotor activity was analysed using the open field apparatus, consisting of a square arena (34 × 34 cm with 24 cm walls) with walls made of translucent plastic. White lines were drawn on the floor and it was divided into nine squares. Mice were placed in the middle of the open field and were allowed to explore the area freely for 6 min. In each trial, the number of times a mouse crossed into an adjacent square (crossing) was recorded. Thigmotaxis (the tendency to stay on the periphery of the open field) was measured to evaluate anxiety-related behaviour.

Preparation of synaptosomes

Purified synaptosomes were prepared by homogenizing tissue in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris, final concentration (f.c.) 0.01 M (Musante *et al.*, 2011). The homogenate was centrifuged at 1000 × *g* for 5 min and the supernatant was stratified on a discontinuous Percoll gradient (2, 6, 10 and 20% v v^{-1} in Tris-buffered sucrose) and centrifuged at 33 500 × *g* for 5 min. The layer between 10 and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellets were resuspended in a physiological solution with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4.

Release experiments

Synaptosomes were incubated for 15 min a 37°C in a rotary water bath in the presence of [^{3}H]-D-aspartate ([^{3}H]-D-Asp, f.c.: 50 nM) or in the presence of [^{3}H]-NA (f.c.: 30 nM); in the latter case 0.1 μM 6-nitroquipazine and 0.1 μM GBR12909 were added to avoid false labelling of 5-hydroxytryptaminergic and dopaminergic terminals respectively. Identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel chambers in a Superfusion System (Raiteri *et al.*, 1974; Ugo Basile; Summa *et al.*, 2013) and maintained at 37°C.

Synaptosomes were transiently (90 s) exposed, at t = 39 min, to high KCl-containing medium (12 mM, NaCl substituting for an equimolar concentration of KCl) in the absence or in the presence of CCL5. Dialyzed 0.1% Polypep was present to avoid sticking of peptides to glass walls and tubing. Fractions were collected as follow: two 3-min fractions (basal release), one before (t = 36–39 min) and one after (t = 45–48 min) a 6-min fraction (t = 39–45 min; evoked release). Fractions collected and superfused synaptosomes were measured for radioactivity.

The amount of radioactivity released into each superfusate fraction was expressed as a % of the total radioactivity. The K^+-induced overflow was expressed as 'induced overflow (%)' and it was estimated by subtracting the neurotransmitter content in the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2).

Quantification of endogenous synaptosomal cAMP

Cortical synaptosomes were first pre-incubated in physiological medium for 5 min at 37°C to equilibrate the system, incubated for 6 min with 12 mM KCl, and then lysed with ice-cold water. Suspensions were centrifuged and supernatants were frozen at –80°C. The endogenous cAMP contents were determined using the Parameter cAMP assay for cAMP quantification (R&D Systems, Minneapolis, MN, USA, Musante *et al.*, 2010). Data are expressed as nmol mg^{-1} protein.

Splenic T-cells isolation and cytokine measurements

Splenocytes were isolated according to Zappia *et al.* (2005) from control and EAE mice at 13 d.p.i. After lysis of red blood cells, splenocytes were plated into 96 well plates at a density of 2×10^5 cells per well in 200 μL RPMI media containing 10% FCS. When indicated, cells were restimulated with hamster monoclonal anti-CD3 (5 μg mL^{-1}) antibody. After 72 h, aliquots of the media were assayed for levels of TNF-α and IL-17 (Quantikine Mouse IL-17 and TNF-α ELISA kits, R&D Systems).

Quantification of endogenous CCL5 and endogenous TNF-α

Serum and cortical tissue samples were obtained from control and EAE mice at 13 d.p.i. Blood samples were collected immediately before animals were killed to harvest CNS tissue. The cortices (about 100 mg wet tissue) were rapidly homogenized at 4°C in 150 μL of the assay diluent RD buffered protein solution and then centrifuged at 15 000 × *g* for 15 min. The endogenous CCL5 levels were measured in the supernatants using the Quantikine Mouse CCL5 ELISA kit (R&D Systems). The amount of chemokine in wet tissue is expressed as pg 100 mg^{-1}. The endogenous TNF-α levels were measured using the Quantikine Mouse TNF-α ELISA kit (R&D Systems). The amount of cytokine in wet tissue is expressed as pg 100 mg^{-1}.

Calculations and statistical analysis

The results were analysed by using ANOVA followed by Dunnett's test or Newman–Keuls multiple comparisons test as appropriate; direct comparisons were performed by Student's *t*-test. Data were considered significant if $P < 0.05$.

Chemicals

[2,3-^{3}H]-D-Asp (specific activity 11.3 Ci mmol^{-1}) was from Perkin Elmer (Boston, MA, USA); 1-[7,8 ^{3}H]-NA (specific activity 39 Ci mmol^{-1}) was from Amersham Radiochemical Centre (Buckinghamshire, UK). The mouse CCL5, Pertussis toxin, Freund's incomplete adjuvant and DMI were from Sigma-Aldrich (Milan, Italy). MOG was from Espikem (Florence, Italy). *M. tuberculosis* (H37Ra) was obtained from DIFCO BACTO Microbiology (Lawrence, KA, USA). Hamster monoclonal anti-CD3 was purchased from eBioscience (San Diego, CA, USA). 6-Nitroquipazine maleate was donated from Duphar, Amsterdam, The Netherlands. GBR12909 was purchased from Tocris Bioscience (Bristol, UK). The drug and molecular target nomenclature conforms to British Journal of Pharmacology's *Guide to Pharmacology* (Alexander *et al.*, 2013).

Results

Clinical signs and endogenous CCL5 production in DMI-treated EAE mice at 13 d.p.i.

Control and EAE mice were randomly assigned to untreated and to DMI-treated groups and CCL5 levels and clinical scores were quantified. In EAE mice, at 13 ± 1 d.p.i., disease onset became evident in the 54.3% of immunized animals (n = 24 mice, see Table 1). Both acute and chronic DMI failed to affect significantly the clinical score in EAE mice when compared with untreated EAE animals (Table 1).

CCL5 endogenous content was quantified in the plasma and in the cortical homogenates of these animals. Table 1 shows that neither acute nor chronic DMI significantly reduced the amount of CCL5 in the plasma of EAE mice at 13 d.p.i. On the contrary, a significant restoration to physiological CCL5 levels in cortical homogenates was detected in acute DMI administered EAE animals (Table 1). Chronic DMI failed to affect the CCL5 overproduction in cortical homogenates from EAE mice at 13 d.p.i. Notably, neither acute nor chronic DMI administration modified on their own the CCL5 levels in the plasma and in cortical homogenates from control mice (Table 1).

[^{3}H]-NA exocytosis from cortical nerve terminals of control and EAE mice at 13 d.p.i.

Cortical synaptosomes from control and EAE mice at 13 d.p.i. were incubated with [^{3}H]NA and then exposed in superfusion to a mild depolarizing (12 mM KCl) stimulus to elicit the Ca^{2+}-dependent, exocytotic-like release of preloaded [^{3}H]NA (Grilli *et al.*, 2008, Figure 1A). Significant changes in the 12 mM K^+-evoked release of tritium could not be observed in EAE mice at 13 d.p.i. when compared with the controls (Figure 1A). The amount of tritium taken up amounted to 21.8 ± 1.85 nCi mg^{-1} synaptosomal protein in control mice (n = 9) and to 25.2 ± 3.06 nCi mg^{-1} synaptosomal protein in EAE mice at 13 d.p.i. (n = 11).

Effects of acute and chronic DMI treatments on the presynaptic α_2-adrenoceptors controlling [^{3}H]-NA exocytosis from cortical nerve terminals of control and EAE mice at 13 d.p.i.

The efficacy of α_2-adrenoceptors in controlling NA exocytosis in *in vitro* superfusion studies was directly quantified by studying the effects of the α_2-adrenoceptor agonist clonidine on the 12 mM KCl-evoked [^{3}H]-NA exocytosis. Clonidine (0.1 μM) potently inhibited the 12 mM K^+-evoked release of [^{3}H]-NA from control and EAE mouse cortical synaptosomes (Figure 1A). At the concentration applied, the agonist was reported to exert the maximal inhibitory effect on these presynaptic receptors (Longordo *et al.*, 2006).

An indirect quantification of the α_2-adrenoceptor-mediated feedback mechanism of control of NA exocytosis was also carried out by quantifying NA exocytosis from terminals isolated from animals administered NA re-uptake

Table 1

Effects of acute and chronic DMI administration on clinical signs and endogenous production of CCL5 in control and EAE mice at 13 d.p.i

	Control mice			EAE mice		
	Control	Acute DMI	Chronic DMI	Control	Acute DMI	Chronic DMI
Clinical score	n.d.	n.d.	n.d.	0.63 ± 0.15	0.32 ± 0.24	0.38 ± 0.26
CCL5 plasma level (pg mL^{-1})	161 ± 37	149 ± 7	146 ± 6	341 ± 46[a]	319 ± 23[b]	291 ± 14[b]
CCL5 level in cortical homogenates (pg 100 mg^{-1} tissue)	128 ± 18	120 ± 28	136 ± 23	603 ± 75[b]	236 ± 43[c]	425 ± 54[b]

Control and EAE mice at 13 ± 1 d.p.i. were administered DMI. DMI acute and chronic treatments were performed as previously described. Clinical signs were detected at 13 ± 1 d.p.i., before mice were killed and are expressed as mean ± SEM. Endogenous CCL5 levels were quantified in the serum of mice at 13 d.p.i and expressed as pg mL^{-1} of serum (at least four mice for each animal group). Endogenous CCL5 levels in cortical homogenates are expressed as pg 100 mg^{-1} wet tissue and they were detected in at least four mice for each different animal group. n.d., not determined.

[a] $P < 0.05$ versus serum CCL5 level in control mice.

[b] $P < 0.01$ versus CCL5 level in cortical homogenates in control mice.

[c] $P < 0.01$ versus CCL5 level in cortical homogenates from EAE mice at 13 d.p.i.

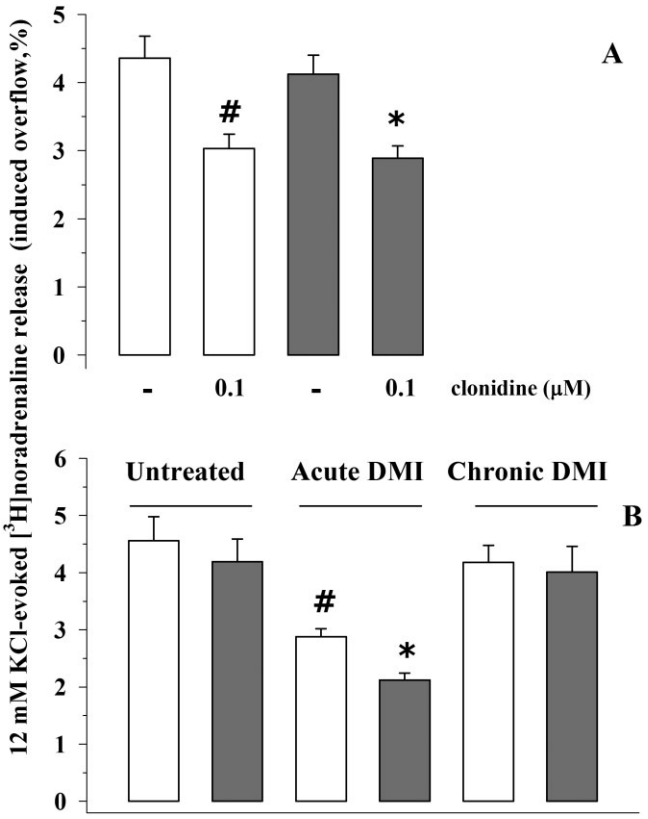

Figure 1

KCl12 mM evoked [^{3}H]-noradrenaline exocytosis from cortical synaptosomes isolated from control and EAE mice at the early stage of the disease: effects of *in vitro* clonidine and *in vivo* DMI. Control (open columns) and EAE (shaded columns) mice were randomly assigned to vehicle and to DMI-treated groups. DMI treatments were carried out as previously described (see Methods section). (A) Effects of *in vitro* clonidine on the 12 mM KCl-evoked [^{3}H]-NA release from synaptosomes isolated from the cortex of control and EAE mice. (B) Effects of acute and chronic *in vivo* DMI administration on the 12 mM KCl-evoked [^{3}H]-NA release from synaptosomes isolated from the cortex of control and EAE mice. Results are expressed as induced overflow; data represent the mean ± SEM of eight experiments run in triplicate. $^{\#}P < 0.05$ versus 12 mM K^+-induced overflow from untreated control mice; $^{*}P < 0.05$ versus 12 mM K^+-induced overflow from EAE-untreated mice at 13 d.p.i.

inhibitors (NRIs) acutely. Our recent observations showed that this event was significantly reduced when compared with that from untreated animals. The likely interpretation of the phenomenon is that, during *ex vivo* isolation, synaptosomes retain the α_2-adrenoceptor-mediated inhibitory input, because the *in vivo* NRI-induced increase in NA bioavailability (Pittaluga *et al.*, 2007) could emerge as decreased tritium release capability in *in vitro* studies. Accordingly, the results in Figure 1B show that acute, but not chronic, *in vivo* DMI caused a significant reduction in amine exocytosis from both control and EAE cortical synaptosomes. Notably, the concomitant *in vivo* administration of the α_2-adrenoceptor antagonist yohimbine prevented the DMI-induced modification to *in vitro* NA exocytosis in control mice (12 mM K^+-evoked [^{3}H]-NA release from control, DMI-untreated mice: 4.33 ± 0.28; 12 mM K^+-evoked [^{3}H]-NA release from acute DMI-treated mice: 2.99 ± 0.38; 12 mM K^+-evoked [^{3}H]-NA release from acute DMI-treated mice injected with yohimbine: 4.01 ± 0.42, results are expressed as induced overflow (data are mean ± SEM, $n = 3$, $P < 0.05$ versus respective control). An almost superimposable reduction in the 12 mM K^+-evoked [^{3}H]-NA release was observed in cortical synaptosomes from EAE mice at 13 d.p.i., which is compatible with the idea that α_2-adrenoceptor-mediated effects were not modified in these animals when compared with controls.

Effects of acute and chronic DMI treatments on [^{3}H]-D-Asp exocytosis from cortical nerve terminals of control and EAE mice at 13 d.p.i.

When cortical synaptosomes isolated from the cortex of control mice and preloaded with [^{3}H]-D-Asp were transiently exposed to a mild, depolarizing stimulus (i.e. 12 mM KCl-containing medium), a significant increase in tritium release occurs; this was halved in EAE mice at 13 d.p.i. (Di Prisco *et al.*, 2013). Acute DMI almost totally prevented this presynaptic impairment, restoring Glu exocytosis (Figure 2) to physiological levels, while chronic DMI administration was ineffective. Both acute and chronic DMI failed to affect on their own [^{3}H]-D-Asp exocytosis in control mice. We asked whether the positive effect acute DMI exerts on presynaptic glutamate defects in the cortex of EAE at 13 d.p.i. could have a protracted effect. To ascertain this, EAE mice were administered DMI acutely at 13 d.p.i. and Glu exocytosis, quantified as [^{3}H]-D-Asp release, was monitored at 21 d.p.i. in cortical synaptosomes. Our data clearly show that the positive effect acute DMI exerts on glutamate exocytosis is retained over time. Actually, the 12 mM K^+-evoked [^{3}H]-D-Asp overflow from cortical synaptosomes isolated from EAE mice at 21 d.p.i., that were acutely administered DMI at 13 d.p.i., was significantly higher than that observed in untreated EAE mice (untreated EAE mouse: 0.60 ± 0.13; DMI-treated mouse: 1.29 ± 0.22; results expressed as induced overflow, data are mean ± SEM; $n = 3$, $P < 0.05$).

Effects of acute and chronic DMI treatments on the presynaptic control CCL5 exerts on [^{3}H]-D-Asp exocytosis from cortical nerve terminals of control and EAE mice at 13 d.p.i.

Nanomolar CCL5 inhibits Glu exocytosis in naive (Di Prisco *et al.*, 2012) and in control mouse (Di Prisco *et al.*, 2013) cortex. CCL5-mediated inhibition of Glu exocytosis, however, turned to facilitation in EAE mice at 13 d.p.i. Figure 2 shows that, in acute DMI-administered EAE mice at 13 d.p.i., CCL5 significantly inhibited the 12 mM K^+-evoked release of [^{3}H]-D-Asp, suggesting that acute drug treatment can restore the CCL5-mediated presynaptic control of Glu exocytosis. Differently from the acute treatment, CCL5-mediated facilitation of glutamate exocytosis was still evident in synaptosomes isolated from the cortex of EAE mice chronically treated with DMI at 13 d.p.i..

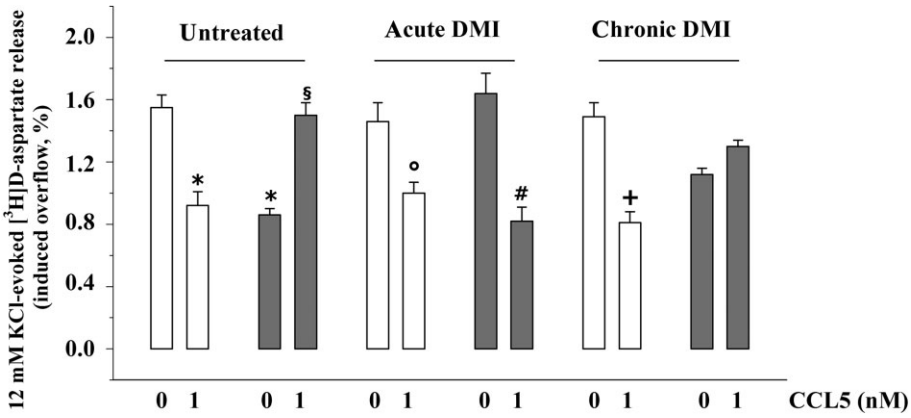

Figure 2

KCl 12 mM evoked [^{3}H]-D-aspartate exocytosis from cortical synaptosomes isolated from control and EAE mice at the early stage of disease: effects of *in vivo* acute and chronic DMI treatments. Control (open columns) and EAE (shaded columns) mice were administered DMI as previously described. Results are expressed as induced overflow; data represent the mean ± SEM of 16 experiments run in triplicate. *$P < 0.05$ versus 12 mM K^+-induced overflow from untreated control mice; $^{§}P < 0.05$ versus 12 mM K^+-induced overflow from EAE untreated mice at 13 d.p.i.; $^{°}P < 0.05$ versus 12 mM K^+-induced overflow from acute DMI-treated control mice; $^{\#}P < 0.05$ versus 12 mM K^+-induced overflow from acute DMI-treated EAE mice; $^{+}P < 0.05$ versus 12 mM K^+-induced overflow from chronic DMI-treated control mice.

Effects of acute and chronic DMI treatments on the cAMP production in cortical nerve terminals of control and EAE mice at 13 d.p.i.

Endogenous cAMP production in synaptosomes isolated from the cortex of EAE mice at 13 d.p.i. was drastically reduced when compared with control (Figure 3, see also Di Prisco *et al.*, 2013), but totally recovered following *in vivo* acute, but not chronic, DMI (Figure 3). In control cortical synaptosomes, acute and chronic DMI failed to affect endogenous cAMP production (Figure 3).

Effects of acute DMI treatment on anxiety and motor behaviour in control and EAE mice at 13 d.p.i.

Behavioural tests were carried out to quantify anxiety and motor performances in control and EAE mice at 13 d.p.i. that were randomly assigned to untreated and to DMI-treated groups. Spontaneous motor behaviour (assessed in open field as number of crossings) was significantly reduced in EAE mice when compared with control and it did not significantly recover in EAE mice administered DMI (Figure 4A, see also Haji *et al.*, 2012). Changes in forced motor score (quantified as mouse performance in the rotarod paradigm, Peruga *et al.*, 2011) in control and EAE mice at 13 d.p.i. were not observed, nor did the administration of DMI in EAE mice affect this behavioural parameter (Figure 4C).

Anxiety-related behaviour (Schiffer and Winemann, 1990) was analysed in the open field paradigm by assessing thigmotaxis (the time spent in the periphery of the open field, Haji *et al.*, 2012). Thigmotaxis was significantly increased in EAE mice at 13 d.p.i., but it significantly recovered in both acute and chronic DMI-treated EAE mice (Figure 4B).

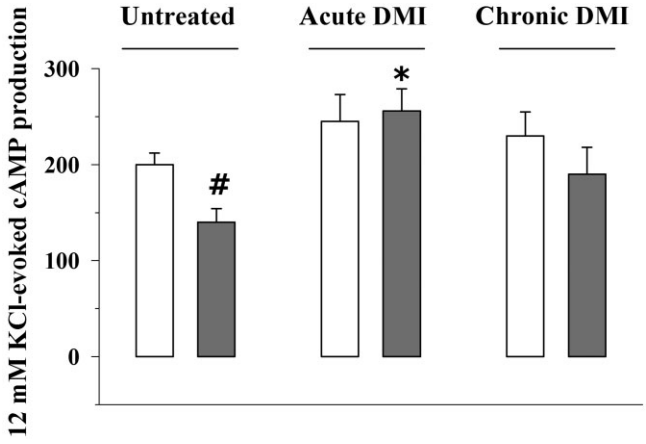

Figure 3

KCl 12 mM evoked endogenous cAMP production in cortical synaptosomes isolated from control and EAE mice at the early stage of disease: effects of *in vivo* acute and chronic DMI treatments. Control (open columns) and EAE (shaded columns) mice were administered DMI as previously described. Results are expressed as nmol mg^{-1} protein; data are mean ± SEM of four experiments run in triplicate. $^{\#}P < 0.05$ versus 12 mM K^+-evoked cAMP production from untreated control mice; *$P < 0.05$ versus 12 mM K^+-evoked cAMP production from untreated EAE mice at 13 d.p.i.

Finally, the explorative behaviour of the EAE mice in a light/dark box analysis was assessed to monitor anxiety-related behaviour. Figure 4D shows that the number of transitions was significantly decreased in EAE mice at 13 d.p.i. when compared with the controls (Figure 4D), and that EAE mice at 13 d.p.i. spent less time in the lighted compartment when compared with the controls (Figure 4E). Interestingly, acute DMI significantly recovered the latter behaviour

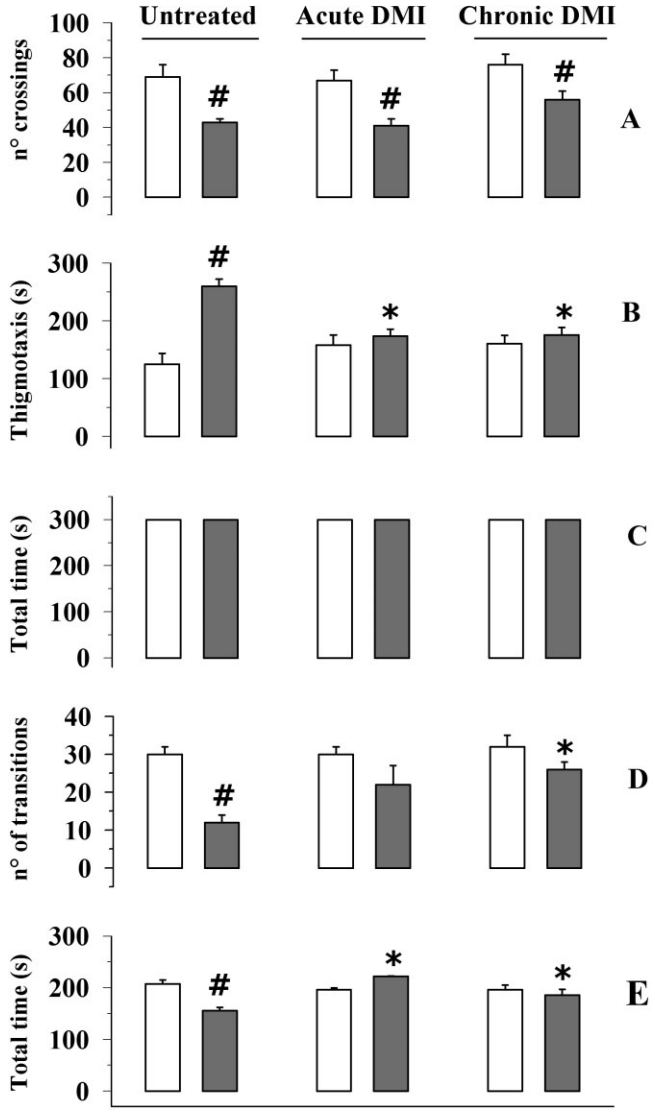

Figure 4

Motor and anxiety behaviour in control and EAE mice at the early stage of disease: effects of *in vivo* acute and chronic DMI treatments. Control (open columns) and EAE (shaded columns) mice were administered DMI as previously described. Spontaneous locomotor activity quantified as number of times a mouse crossed into an adjacent square (n° crossings, A). In the same experimental paradigm, anxiety was quantified as time spent in periphery (thigmotaxis, B). Forced motor activity was quantified as the latency to fall from the rod set at a fixed speed of 16 r.p.m. for a maximum of 300 s (C). Explorative and anxiety-related behaviour were quantified in the light dark box as total time spent into the lighted compartment of the light dark box (total time, E) and as number of transitions (n° transitions, D). Data represent the mean ± SEM of five mice for each group of animals. $^{\#}P < 0.05$ versus respective control; $^{*}P < 0.01$ versus untreated EAE mice at 13 d.p.i.

(Figure 4E), but not the number of transitions (Figure 4D). In contrast, in EAE mice at 13 d.p.i., chronic DMI restored both the permanence in the light side and the number of transitions (Figure 4D and E) to physiological levels when compared with untreated EAE mice.

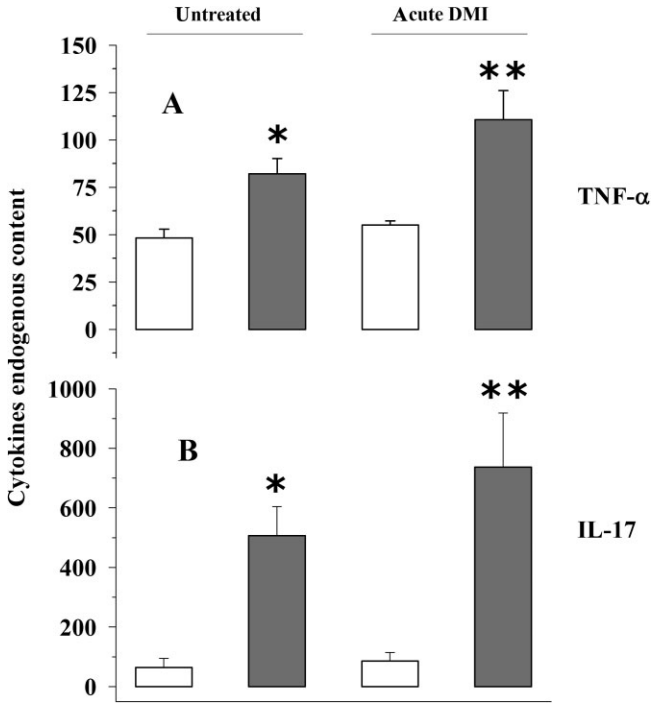

Figure 5

Endogenous cytokine levels in splenocytes isolated from control and EAE mice at the early stage of disease: effects of *in vivo* acute DMI treatment. Control (open columns) and EAE (shaded columns) mice were administered DMI as previously described, Splenic T-cells were activated by anti-CD3 antibody and the supernatant was analysed for TNF-α (A) and IL-17 (B) endogenous content. The results are expressed as pg mL^{-1} supernatant. Data represent the mean ± SEM of four mice for each group of animals. $^{*}P < 0.05$ versus respective control; $^{**}P < 0.001$ versus respective control.

Effects of acute DMI treatment on peripheral TNF-α and IL-17 production in EAE mice at 13 d.p.i.

Experiments were carried out to evaluate whether peripheral events could have a role in the DMI-induced restoring activity. With this aim, splenic T-cells from DMI-treated and untreated control and EAE mice at 13 d.p.i. were restimulated with anti-CD3 antibody and the endogenous production of the pro-inflammatory cytokines TNF-α and IL-17 was quantified in the conditioned media generated after a 72 h incubation. TNF-α (Figure 5A) endogenous production was largely increased in splenocytes isolated from control and EAE mice that were exposed to an anti-CD3 antibody when compared with cytokine production from untreated cells (basal TNF-α content in control splenocytes: 20.6 ± 2.7 pg mL^{-1}; basal TNF-α content in EAE mouse splenocytes: 21.7 ± 4.7pg mL^{-1}; respectively, compare with data in Figure 5A). *In vivo* acute DMI failed to prevent the cytokine overproduction from both control and EAE T-cells (Figure 5A). IL-17 production was not significantly modified in anti CD3-treated splenocytes from control mice but it was significantly increased in the supernatant of anti CD3-treated T-cells from EAE mice (basal IL-17 content in control splenocytes: 15.4 ± 0.73 pg mL^{-1}; basal IL-17 content in EAE mouse splenocytes: 19.79 ±

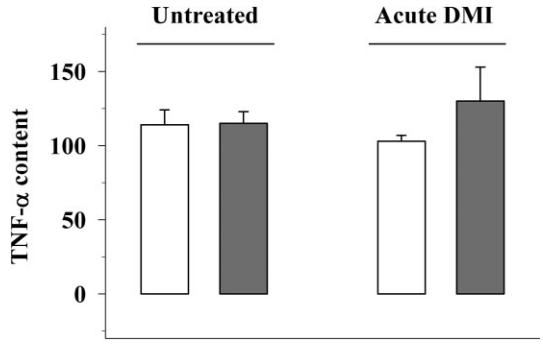

Figure 6

Endogenous TNF-α content in cortical homogenates from Control (open columns) and EAE (shaded columns) mice at the early stage of disease. The amount of endogenous TNF-α was expressed as pg 100 mg^{-1} wet tissue. Data are expressed as mean ± SEM of four mice for each group of animal.

2.88 pg mL^{-1} respectively, Figure 5B). *In vivo* acute DMI failed to prevent the IL-17 overproduction from EAE T-cells (Figure 5B).

Cortical endogenous TNF-α production in EAE mice at 13 d.p.i.: effect of acute DMI

Recent studies have shown that the endogenous production of TNF-α increases in selected CNS regions during EAE (Haji *et al.*, 2012; Acharjee *et al.*, 2013) but data concerning the cortex of EAE at 13 d.p.i. are, so far, lacking. As illustrated in Figure 6, significant differences could not be detected in EAE mouse cortex when compared with control, and acute DMI did not affect the TNF-α production in these animals.

Discussion

The present work aimed to assess whether strengthening NA transmission at the early presymptomatic stage of EAE could lessen central CCL5 overproduction and exert beneficial effects (Simonini *et al.*, 2010) on the presynaptic neuronal defects that occur in the cortex of almost asymptomatic EAE mice (Di Prisco *et al.*, 2013). Before verifying this hypothesis, it was important to ascertain whether noradrenergic transmission could be altered at this stage of disease. We therefore analysed some functional parameters of the noradrenergic system in the cortex of EAE mice at 13 d.p.i. (i.e. when cortical glutamatergic nerve terminals were reported to undergo severe defects, Di Prisco *et al.*, 2013). The first observation of the present study was that cortical [^{3}H]-NA release capability as well as synaptosomal labelling, that represent an indirect measure of monoamine uptake efficiency, were unchanged in the cortex of EAE mice at 13 d.p.i. In as much as, the efficacy of presynaptic α_2-adrenoceptors in controlling NA exocytosis was unaltered, leading us to propose that, unlike the glutamatergic system, perturbations of cortical noradrenergic transmission at the early asymptomatic stage of disease are rare, if ever significant. Rather, our results suggest that the noradrenergic pathway is unaltered, giving the rationale for the use of drug(s) tuning NA metabolism and/or uptake (NRIs as well as monoaminooxidase inhibitors, O'Sullivan *et al.*, 2009; Vollmar *et al.*, 2009; Musgrave *et al.*, 2011) in MS therapy.

The finding that the efficacy of presynaptic α_2-adrenoceptors was unmodified in EAE mice at 13 d.p.i. deserves particular attention if one considers that, beside their well-documented role as auto-heteromodulator of central neurotransmission (Langer, 1981; 2008; Starke *et al.*, 1981; Pittaluga and Raiteri, 1988; Wang *et al.*, 2002), these receptors also mediate NA-induced anti-inflammatory properties. In particular, increased NA bioavailability that follows DMI treatment was reported to inhibit CCL5 overexpression (O'Sullivan *et al.*, 2010) triggered by pro-inflammatory stimuli, through a cascade of molecular events involving either direct (Szelenyi and Vizi, 2007; O'Sullivan *et al.*, 2010) or indirect (involving TNF-α as an intermediate, Reynolds *et al.*, 2005) α_2-adrenoceptor-mediated pathways. In line with these observations, the second finding of the present work was that, in the cortex of EAE mice at the early stage of disease, DMI significantly reduced CCL5 overexpression. Quite surprisingly, acute administration of the DMI was efficacious in this brain region, while chronic treatment was inactive. The huge discrepancy between acute and chronic DMI seems well consistent with the above-mentioned involvement of α_2-adrenoceptor-mediated events. Actually, when chronically activated by the endogenous amine, because of the prolonged blockade of NA transporters, α_2-adrenoceptors are down-regulated (Raiteri *et al.*, 1983; Pittaluga *et al.*, 2007) so that their cellular effects are silenced. In other words, here, we propose that the lack of efficacy of chronic DMI in our experimental conditions could be due to DMI-induced desensitization of the α_2-adrenoceptors controlling CCL5 expression.

DMI-induced anti-inflammatory activity was restricted to the CNS, as its acute administration did not modify the peripheral productions of IL-17 and TNF-α, or reduce CCL5 plasma levels. The striking difference between the periphery and CNS could imply that the migration of peripheral pro-inflammatory products into the CNS was impeded because of the integrity of the blood–brain barrier or because of the reduced expression of cell adhesion molecules elicited by the DMI treatment itself (O'Sullivan *et al.*, 2010). Accordingly, in presymptomatic EAE mice, changes in cortical TNF-α levels could not be observed, also favouring the hypothesis that a direct α_2-adrenoceptor-mediated pathway could better account for the DMI-induced reduction in CCL5 overexpression in EAE mouse cortex.

Recently, we hypothesized that increased CCL5 bioavailability in the CNS could have a role in the onset of the presynaptic alterations in EAE mice (Di Prisco *et al.*, 2013). Well in line with our hypothesis, the third finding of the present work was that in acute DMI-treated EAE mice at 13 d.p.i., when the cortical CCL5 endogenous level was significantly reduced, the altered release capability in glutamatergic nerve endings and the impaired endogenous production of cAMP in cortical synaptosomes were restored to physiological levels, while the shift from inhibition to facilitation of the presynaptic control CCL5 exerts on Glu exocytosis (Di Prisco *et al.*, 2013) did not take place. Unexpectedly, and in a way quite surprisingly, acute DMI-induced restoration of gluta-

mate exocytosis was still detectable 1 week after drug treatment, at 21 d.p.i. (i.e. the acute stage of disease), suggesting that DMI-induced modifications lasted a long time. Further studies are required to investigate the effect of acute early DMI on disease progression. Recent evidence has highlighted the role of glutamate in anxiety and mood disorders and several compounds with antidepressant activity are known to restore glutamatergic transmission to physiological levels (Bonanno *et al.*, 2005; Marrocco *et al.*, 2013; Nasca *et al.*, 2013). The DMI-induced restoration of glutamate exocytosis might therefore well account for the decreased anxiety-like behaviour observed in EAE mice at 13 d.p.i.

Glu exocytosis is a Ca^{2+}-dependent process that partly depends on AC/cAMP/ PKA-mediated processes (Grilli *et al.*, 2004; Musante *et al.*, 2008; Di Prisco *et al.*, 2012). Although the heterogeneity of the cortical synaptosomal fraction must be taken in consideration, glutamatergic synaptosomes represent about the 40–50% of the entire synaptosomal population. Because of this large percentage, a tight correlation between Glu release and endogenous cAMP production could be proposed to exist, so that the reduced cAMP production observed in cortical synaptosomes from presymptomatic EAE mice at 13 d.p.i. would also be expected to occur in glutamatergic nerve endings.

The impaired AC activity detected in EAE mice at the early stage of disease (Di Prisco *et al.*, 2013) might have a major role in the onset of presynaptic defects. Actually, it could account for decreased efficiency in Glu exocytosis, but it also might favour the unusual coupling of chemokine receptors (CCRs) to enzymatic pathway(s) other than AC, then giving a rationale for the shift from inhibition to facilitation of the presynaptic CCL5-mediated control of Glu exocytosis (Di Prisco *et al.*, 2013). The possibility should be therefore considered that, beside the increased CCL5 overproduction, the drastic reduction of cAMP content in cortical synaptosomes might also represent a crucial event accounting for the presynaptic defects observed in asymptomatic EAE mice at 13 d.p.i. If this is the case, the DMI-induced normalization of cAMP production would be expected to favour the restoration of presynaptic functions.

The beneficial effect of the AC/cAMP enzymatic pathway in demyelinating disease has already been investigated and increasing cAMP levels with phosphodiesterase inhibitors (Genain *et al.*, 1995; Sommer *et al.*, 1997) was shown to ameliorate disease progression in EAE mice. As an alternative to the use of phopshodiesterase inhibitors, here we demonstrate that acute DMI also favours this enzymatic pathway, and ameliorates neuronal defects and anxiety-related behaviours in EAE mice.

Several mechanisms might account for the DMI-induced restoration of endogenous cAMP production. By reducing CCL5 expression, DMI can prevent the *in vivo* pathological overactivation of presynaptic inhibitory CCRs (Di Prisco *et al.*, 2012), thus favouring the accumulation of cAMP in glutamatergic nerve endings. Concomitantly, by acting at β_2-adrenoceptors presynaptically located on glutamatergic nerve endings, the acute DMI-induced increase in NA bioavailabililty would also favour cytosolic cAMP accumulation in these terminals (Wang *et al.*, 2002). Finally, the increased production of neuroprotective endogenous factors such as brain-derived neuronal factor that usually follows NRI-administration could increase the expression of transcription factors including cAMP (Vollmar *et al.*, 2009 and references therein).

Again, as already observed for central CCL5 overproduction, chronic DMI was both ineffective at restoring cAMP production and glutamate release, and at presynaptic CCR-mediated control of glutamate exocytosis. Also in this case, the huge discrepancy between acute and chronic DMI-mediated effects seems compatible with the idea that adrenoceptor-mediated effects could have a major role in the DMI-induced positive effects. In the present case, β_2-adrenoceptor-mediated effects also deserve attention. In fact, in addition to α_2-adrenoceptors, presynaptic β_2-adrenoceptors located on glutamatergic nerve endings, also desensitize when chronically exposed to increased extracellular levels of NA (Nalepa *et al.*, 1998).

Besides the well-known role of cortical noradrenergic and glutamatergic transmission in mood disorders (Pittaluga *et al.*, 2007; Marrocco *et al.*, 2013; Nasca *et al.*, 2013), recent evidence strongly supports the notion that depression and anxiety is an inflammatory condition and that elevated levels of endogenous cytokines (including TNF-α, Hashmi *et al.*, 2013) might have a role in eliciting these behaviours. Whether pathologically-relevant amounts of CCL5 could participate in determining anxiety and depression in MS patients remains to be established, although the positive effects acute DMI exerts on thigmotaxis (being inactive on motor behaviour and clinical scores) could support this hypothesis.

To conclude, our results demonstrate that acute DMI exerts a beneficial effect on the early neurological defects observed in EAE mice. Notably, other drugs, including metformin and mitoxantrone derivatives (Nath *et al.*, 2009; Alves *et al.*, 2012) have been reported to control CCL5 overproduction. Our study, therefore, sheds light on the molecular event(s) involved in the efficacy of a heterogeneous class of therapeutics sharing a common mechanism of action that could represent a useful approach to restrain neurological MS symptoms. Interestingly, chronic DMI administration in MS patients suffering major depressive disorders was limited because of DMI-induced side effects (Schiffer and Wineman, 1990). The use of acute administration should bypass this problem, allowing the use of this antidepressant for the cure of MS symptoms.

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Conflict of interest

None.

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