Neurobiology of Disease 74 (2015) 347–358

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Dopaminergic dysfunction is associated with IL-1β-dependent mood alterations in experimental autoimmune encephalomyelitis

Antonietta Gentile [a,b,1], Diego Fresegna [a,1], Mauro Federici [a], Alessandra Musella [a,b], Francesca Romana Rizzo [a], Helena Sepman [a,b], Silvia Bullitta [a], Francesca De Vito [a], Nabila Haji [a], Silvia Rossi [a,b], Nicola B. Mercuri [a,b], Alessandro Usiello [c,d], Georgia Mandolesi [a], Diego Centonze [a,b,*]

[a] Fondazione Santa Lucia/Centro Europeo per la Ricerca sul Cervello (CERC), Via del Fosso di Fiorano 64, 00143 Rome, Italy
[b] Clinica Neurologica, Dipartimento di Medicina dei Sistemi, Università Tor Vergata, Via Montpellier 1, 00133 Rome, Italy
[c] Behavioural Neuroscience Laboratory, CEINGE — Biotecnologie Avanzate, Via Comunale Margherita 482, 80145 Naples, Italy
[d] Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples (SUN), Caserta, Italy

ARTICLE INFO

Article history:
Received 22 September 2014
Revised 21 November 2014
Accepted 26 November 2014
Available online 12 December 2014

Keywords:
EAE
IL-1β
Striatum
Dopamine
Depression

ABSTRACT

Mood disturbances are frequent in patients with multiple sclerosis (MS), even in non-disabled patients and in the remitting stages of the disease. It is still largely unknown how the pathophysiological process on MS causes anxiety and depression, but the dopaminergic system is likely involved.

Aim of the present study was to investigate depressive-like behavior in mice with experimental autoimmune encephalomyelitis (EAE), a model of MS, and its possible link to dopaminergic neurotransmission. Behavioral, amperometric and biochemical experiments were performed to determine the role of inflammation in mood control in EAE. First, we assessed the independence of mood alterations from motor disability during the acute phase of the disease, by showing a depressive-like behavior in EAE mice with mild clinical score and preserved motor skills (mild-EAE). Second, we linked such behavioral changes to the selective increased striatal expression of interleukin-1beta (IL-1β) in a context of mild inflammation and to dopaminergic system alterations. Indeed, in the striatum of EAE mice, we observed an impairment of dopamine (DA) neurotransmission, since DA release was reduced and signaling through DA D1- and D2-like receptors was unbalanced.

In conclusion, the present study provides first evidence of the link between the depressive-like behavior and the alteration of dopaminergic system in EAE mice, raising the possibility that IL-1β driven dysfunction of dopaminergic signaling might play a role in mood disturbances also in MS patients.

Introduction

Multiple sclerosis (MS) is a frequent inflammatory and demyelinating disease of the central nervous system (CNS), and the main cause of non-traumatic disability in young adults (Compston and Coles, 2008). Clinical assessment of MS patients is generally directed to the identification of physical symptoms like motor and sensory deficits, and their prevention is the main objective of treatment with the currently approved disease-modifying pharmacological agents.

It is now clear that MS is a diffuse disease of the CNS, involving since its early phases both white and cortical and subcortical gray matter (Geurts and Barkhof, 2008). In this respect, the discovery that cognition is affected by the disease even in otherwise stable patients was a first clinical indication that MS causes not only white matter demyelinating lesions but also widespread neuronal dysfunction.

Also anxiety and depression are frequent in MS, even in non-disabled patients and in the remitting stages of the disease (Feinstein, 2007), and they are generally undervalued and undertreated in clinical practice (Marrie et al., 2009). Poor knowledge of the pathophysiological mechanisms of mood alteration in MS could explain the scarce attention paid to this potentially serious comorbid condition. Mood disorders are in fact commonly viewed as reasonable reactions to physical limitations or to worries of disease progression in MS patients (Brown et al., 2009), but recent data suggest that immune molecules, such as IL-1β and TNF-α, can alter mood in neuroinflammatory diseases even independently of their destructive effects on brain tissue and therefore motor disability (Haji et al., 2012; Peruga et al., 2011; Pollak et al., 2002).

The dopaminergic system is recognized as an important regulator of mood and several data suggest the impairment of dopaminergic neurotransmission during neuroinflammatory conditions (Felger and Miller, 2012). Dopaminergic dysfunction has been described also in MS (Markianos et al., 2009) and in EAE (Benson et al., 2013). The

* Corresponding author at: Clinica Neurologica, Dipartimento di Medicina dei Sistemi, Università Tor Vergata, Via Montpellier 1, 00133 Rome, Italy. Fax: +39 06 7259 6006.
E-mail address: centonze@uniroma2.it (D. Centonze).
[1] AG and DF are equally contributing first authors.
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2014.11.022
0969-9961/

basal ganglia are key subcortical structures that regulate motivation and motor activity and dopamine plays an essential modulatory role in its function. Of note, the striatum, which is part of the basal ganglia, is involved in both EAE (Centonze et al., 2009; Rossi et al., 2010) and MS (Bermel et al., 2003; Tao et al., 2009).

Moving from this evidence, aim of the present investigation was to uncover the molecular and synaptic bases of mood alterations in EAE, and the possible involvement of the dopaminergic system. In an attempt to differentiate the mechanisms of behavioral alterations from those leading to neurological dysfunction, depression-like symptoms were explored in EAE independently of motor impairment during the acute phase of the disease.

Material and methods

Animals

C57BL/6 mice (Charles-River, Italy) were randomly assigned to standard cages (4–5 animals per cage) and kept at standard housing conditions, including a Techniplast Mouse House®, with a light/dark cycle of 12 h and free access to food and water. Minipump-implanted mice were housed in isolated cages endowed with special bedding (TEK-FRESCH, Harlan). Beginning one week before the immunization, all animals were daily kindly handled to reduce the stress induced by operator manipulation during behavioral experiments.

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November, 1986 (86/609/EEC).

EAE induction and clinical evaluation

EAE was induced in 7–8 weeks female C57BL/6 animals as previously described (Centonze et al., 2009; Mandolesi et al., 2013). Control animals received the same treatment as EAE mice without the immunogen MOG peptide (referred to as 'CFA'). The animals were daily scored for clinical symptoms of EAE, according to the following scale: 0 = healthy; 1 = flaccid tail; 2 = ataxia and/or paresis of hindlimbs; 3 = paralysis of hindlimbs and/or paresis of forelimbs; 4 = tetraparalysis; 5 = moribund or death due to EAE. Intermediate clinical signs were scored adding 0.5 value (Aktas and Zipp, 2003; Centonze et al., 2009; Mandolesi et al., 2013). Also CFA mice were daily manipulated, to avoid the influence of different handling on behavioral outcomes among experimental groups. In EAE mice, first clinical symptoms appear about 10–12 days post immunization (dpi) with a peak of severity at about 20–25 dpi: this stage is referred to as symptomatic or acute phase of the disease (Centonze et al., 2009). The average number of MOG-immunized animals was 45 ± 5. At 20–25 dpi, MOG-immunized animals were divided into two groups, according to clinical score: mice with score < 1 (herein referred to as mild-EAE) and EAE mice with score ≥ 1. The percent of mild-EAE mice was about 20 for each immunization.

Minipump implantation and continuous intracranial infusion

One week before immunization, mice were implanted with a minipump in order to allow continuous intracerebroventricular (icv) infusion of either vehicle or interleukin 1 receptor antagonist (IL-1ra) (150 ng/day; R&D Systems) for 4 weeks (3 sets of immunization) (Mandolesi et al., 2013).

Behavioral assessment

The animals were tested during the light period (9:00–12:00 am) in a dedicated room with a constant temperature (26 ± 1 °C). All tests were performed in different days with distinct groups of animals. Each session was preceded by at least 1 h habituation in the behavioral room. Animals' behavior was analyzed by trained observers blind to treatment and experimental group.

Rotarod (RR)

RR analysis is one of the most widely used tests to assess motor coordination in rodents. The latency to fall was measured on a rotating rod at an accelerating speed from 4 to 40 rotations/min in 300 s (Harvard Apparatus, UK). After a training phase, the time and speed at which the mouse fell off the rod was recorded. Each mouse from the three experimental groups (EAE mean score 2.5 at 21 dpi n = 5; mild-EAE score < 1 n = 10; CFA n = 11) had three trials and the mean latency to fall per trial was calculated. Since mice with a score > 2.0 were frequently unable to brace their fall from the rod, cushioning material was placed so that the height of the fall was decreased from 45 to approximately 20 cm.

Grip strength test

Immunized mice were tested for grip strength performance using the Grip Strength Meter (Ugo Basile, Italy). The apparatus for this test consisted of a steel wire grid (8 × 8 cm) connected to an isometric force transducer. Mice were lifted by their tail so that they grasped the grid with their paws. Mice from the three experimental groups (EAE mean score 2.5 at 21 dpi n = 5; mild-EAE score < 1 n = 10; CFA n = 11) were then gently pulled backward until they released the grid and the maximal force in newtons (N) exerted by the mouse before losing the grip was measured. The mean of three consecutive measurements for each animal was calculated.

CatWalk gait analysis

Mice from the three experimental groups (EAE mean score 2.5 at 21 dpi n = 5; mild-EAE score < 1 n = 10; CFA n = 11) were subjected to gait assessment with the CatWalk automated gait analysis system (Noldus Information Technology, Wageningen, The Netherlands) (Hamers et al., 2006). The apparatus comprises a long glass plate with a fluorescent light beamed into the glass walkway floor from one side. In a dim environment, the light is reflected downward and the footprints of the mouse are recorded by a camera mounted under the glass, as the mouse walks along the walkway. Contact areas are automatically indexed and interactively assigned colored tags (e.g., left/right fore-/hindpaw, nose, abdomen and tail), which allow quantitative analysis of gait: step (stride length), swing (amount of time between one step and the next on a particular paw), stance (the amount of time a particular paw is on the walkway), base of support (the distance between the two forepaws and the two hindpaws), paw contact area, and intensity (the intensity of light of a particular paw print). Some paw parameters could be in part influenced by the body weight of the mice; of note, a 15–20% loss of body weight, which is typical for EAE mice, was not observed in mild-EAE mice. The animals were trained to make runs on the walkway with a stable crossing time: EAE mice due to their motor disability were slower in comparison to both CFA and mild-EAE mice. This could in part affect velocity-dependent parameters, like the regularity index and the duty cycle, in EAE mice. At least three uninterrupted runs per animal were used for testing the mice. The following parameters were analyzed:

- Regularity index (RI), which expresses the number of normal step sequence patterns relative to the total number of paw placements. It is a percent index and is used as a measure of the degree of inter-limb coordination during the gait cycle.
- Base of support (BOS), which is an indication of double-limb support. It is derived by measuring the distance (mm) between the mass midpoints of the two forelimb prints or the two hindlimb prints at maximum contact.
- Max contact max intensity, that is the maximum intensity at max contact of a paw. Intensity ranges from 0 to 255. The intensity of a print depends on the degree of contact between a paw and the

glass plate and increases with increasing pressure. Therefore, Intensity is a measure of pressure exerted on the glass plate.
- Print length, that is the length (horizontal direction) of the complete print.
- Stride length, which is the distance (in distance units) between successive placements of the same paw.
- Print area, which is the surface area (in the distance unit) of the complete print.
- Duty cycle, which expresses stance duration as a percentage of the duration of the step cycle. It is calculated as follows: duty cycle: stance/stance + swing * 100%.

Nest building (NB)

To evaluate the quality of nest construction, acute-phase mice (n = 7 per group) were individually housed 1 h before the onset of dark phase in a clean cage overnight with no enrichment aside a pre-weighted roll of cotton in the cage-top food hopper. When EAE mice were tested during the acute phase, we lowered the distance between the cotton in the cage-top food hopper and the flat of the cage by adding the same amount of sawdust in each cage. The morning after the quality of the nest was evaluated using the following scoring system: (1) no nest, (2) platform-type nest consisting of a pallet on the floor of the cage, (3) bowl- or cup-shaped nest with sides, or (4) bowl- or cup-shaped nest with sides and a cover (Bulloch et al., 1982).

Forced swimming test (FST)

FST was performed only in animals with mild EAE phenotype (n > 7 for all experimental groups). Each mouse was placed into a 5-l glass beaker (height 23.5 cm; diameter 16.5 cm) containing water up to a height of 15 cm at 25 ± 1 °C for 6 min. The water was exchanged after each trial. Floating (immobility) and struggling time were scored over the entire 6 min exposure by pressing pre-set keys on a computer keyboard, using customized freeware software (ODLOG). Immobility was estimated during the last 4 min of the test, because mobility of animals is increased during first minutes of water immersion. A mouse was judged floating when it stopped any movements except those that are necessary to keep its head above water.

Tail suspension test (TST)

TST was performed only in animals with mild EAE phenotype (n > 7 for all experimental groups). Mice were secured to a piece of suspended tubing by adhesive tape placed approximately 1 cm from the tip of the tail and were suspended 50 cm from the benchtop within a visually isolated area for 6-min test session. Since little immobility is observed during first two minutes, the duration of immobility was estimated during the last 4 min of the test, using customize freeware software (ODLOG). An animal was rated as immobile when there was no movement of the head, extremities or the torso.

Constant potential amperometry (CPA)

Preparation and maintenance of corticostriatal slices have been previously described (Mercuri et al., 1995). Amperometric detection of DA was obtained by using carbon fiber electrodes (World Precision Instruments GmbH, Germany) positioned to a depth of 50–150 μm near the bipolar Ni/Cr stimulating electrode into the corticostriatal slices of mice.

Constant potential amperometry was obtained with a World Precision Instruments MicroC, holding the electrodes at an oxidation potential of 0.55 to 0.60 V versus a reference electrode Ag/AgCl (Kawagoe and Wightman, 1994). Currents sampled at this oxidation peak potential were measured to provide profiles of DA concentration versus time. Electrode calibration was performed at the end of each experiment by bath perfusing DA (0.3–10 μM). For stimulation protocol, we applied a single rectangular electrical pulse using a Digitimer DS3 Stimulator every 3 min along the range of stimulation intensity (20–1000 μA, 20–40 μs duration). Signals were digitalized using a Digidata acquisition system (Digidata 1440A) coupled to a PC running the Clampex 10.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Twenty-one dpi striata and hippocampi of EAE, mild-EAE and CFA mice (at least 5 for each group) were dissected (see western blot section). Amygdalae were dissected from fresh brain sections (900 μm) by means of the tip of a Pasteur pipette in cold artificial cerebro-spinal fluid (ACSF) solution (the same condition of CPA experiments). All the procedures were performed in RNAse-free conditions. Total RNA was extracted according to the standard miRNeasy Mini kit protocol (Qiagen). The RNA quantity and purity were analyzed with the Multiskan Go microdrop Plate spectrophotometer (Thermo Scientific). The quality of RNA was assessed by visual inspection of the agarose gel electrophoresis images. Next, 200–500 ng of total RNA was reverse-transcribed using miScript II RT Kit (Qiagen) according to the manufacturer's instructions; 10–50 ng of cDNA was amplified with SensiMix SYBR Hi-Rox Kit (Bioline; Meridian Life Science) in triplicate using the Applied Biosystem 7900HT Fast Real Time PCR system. Relative quantification was performed using the ddCT method. β-actin was used as internal controls. The following primer sequences were used.

IL-1β (NM_008361): 5′-GGACCTTCCAGGATGAGGACAT-3′ (sense); 5′-GCTCATGGAGAATATCACTTGTTGG-3′ (antisense); TNF-α (NM_013693): 5′-CCTCTTCTCATTCCTGCTTGTGG-3′ (sense); 5′-ACTTGGTGGTTTGCTACGACG-3′ (antisense); β-actin (NM_007393): 5′-CCTAGCACCATGAAGATCAAGATCA-3′ (sense); 5′-AAGCCATGCCAATGTTGTCTCT-3′ (antisense).

Immunohistochemistry and confocal microscopy

The immunofluorescence experiments were performed similarly to a method described previously (Mandolesi et al., 2013). Mice at least from 2 to 3 different immunization experiments were killed at 21 dpi. The following primary antibodies were used: goat anti-IL-1β (1:200; R&D Systems), rabbit anti-Iba1 (1:750; Wako Chemicals, USA). These antibodies were used in combination with the following secondary antibodies: Alexa Fluor-488 (1:200; Life Technologies) and Cy3-conjugated donkey anti-rabbit or anti-goat (1:200; Jackson ImmunoResearch). DAPI (0.01 mg/ml) was used to visualize nuclei. For the specificity of the antibodies see Mandolesi et al. (2013).

Images were acquired using a LSM7 Zeiss confocal laser-scanner microscope with 10× (zoom 0.5×) or 63× oil objective (NA: 1.4; zoom: 0.5×). The images had a pixel resolution of 1024 × 1024. The confocal pinhole was kept at 1.0, the gain and the offset were lowered to prevent saturation in the brightest signals, and sequential scanning for each channel was performed. Z-stack images were acquired, z-projected (63 × 0.5×; z-step: 2 μm; 10 × 0.5×; z-step: 10 μm), and exported in TIFF file format and adjusted for brightness and contrast as needed, using ImageJ software. Smooth filter was used to reduce noise. All qualitative analyses were performed on at least four images acquired from at least four serial sections (for each brain area) per animal (n = 4 each group) from at least two independent experiments.

Striatal total protein extracts preparation and western blot (WB)

At least 4 animals per group were included in all WB experiments. Twenty-one dpi mice were sacrificed through cervical dislocation and both left and right striata were quickly removed and frozen until use. Tissues were homogenized in RIPA buffer plus protease and phosphatase inhibitors cocktail (SIGMA) as previously described (Mandolesi et al., 2013).

Primary antibodies were used as following: mouse anti-β-actin (1:20,000, 1 h RT; Sigma-Aldrich), rabbit anti-tyrosine hydroxylase (TH: 1:2000, overnight +4 °C; Abcam), rabbit anti-DA D2R (1:1000

diluted in 5% BSA, overnight + 4 °C; Millipore), rat anti-DA D1R (1:2000, 2 h RT; Sigma-Aldrich), rabbit anti-DARPP32 (1:50,000, 1 h RT; Abcam), rabbit anti-p-Th34-DARPP32 (1:1000, overnight + 4 °C; Merck), anti-p-Th75-DARPP32 (1:1000, overnight + 4 °C; Merck). Membranes were incubated with the following secondary antibodies: anti-mouse IgG HRP (1:10,000; GE Healthcare, formerly Amersham Biosciences), anti-rabbit IgG HRP (1:2000; GE Healthcare, formerly Amersham Biosciences), anti-rat IgG HRP (1:2000; GE Healthcare, formerly Amersham Biosciences), diluted in 1% milk for 1 h at RT. After detection of phospho-sites, blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific); complete stripping was assessed by incubation with proper secondary antibody immunodetection was performed by ECL reagent (Amersham) and membrane was exposed to film (Amersham). Densitometric analysis of protein levels was performed by NIH ImageJ software (http://rsb.info.nih.gov/ij/). WB results are presented as data normalized to control CFA values.

Statistical analysis

For each type of experiment and time-point, at least four mice per group were employed. For behavioral tests at least seven mice per group were analyzed. Throughout the text "n" refers to the number of animals, except for CPA experiments, where it means the number of the recorded corticostriatal slices. Data were presented as the mean ± S.E.M. The significance level was established at $p < 0.05$. Statistical analysis was performed using unpaired Student's *T*-test for comparisons between two groups and non-parametric Mann–Whitney test. Multiple comparisons were analyzed by one-way ANOVA for independent measures followed by Tukey's HSD or by Kruskal–Wallis test followed by Dunn's post hoc analysis for non-parametric values.

Results

EAE causes depressive-like behavior independently of motor deficits

To investigate the molecular link between depressive-like behavior and inflammation in the EAE model in the acute phase of the disease, we first wanted to exclude the effects of substantial motor impairment on behavioral tests. To this aim, when immunized mice reached the peak of the disease, we classified EAE mice according to their clinical score (mild-EAE with score < 1 and EAE with score ≥ 1, Fig. 1A) and we assessed neuromuscular (grip-strength test; Murphy et al., 2012) and motor performances (rotarod, and CatWalk gait analysis) on these mice and their relative controls. Mild-EAE mice occur with a frequency of 20% throughout the present study. As expected, rotarod performance mirrored the EAE score: EAE mice were almost unable to perform this test and fell off the rod immediately after it began turning, while mild-EAE mice showed latency values similar to CFA group (Fig. 1B; CFA n = 11, EAE n = 5, mild-EAE n = 10; one-way ANOVA: CFA vs EAE $p < 0.001$; EAE vs mild-EAE $p < 0.001$). Similar results were obtained for the grip strength test, suggesting that the neuromuscular function of mild-EAE was not affected (Fig. 1C; CFA n = 11, EAE n = 5, mild-EAE n = 10; one-way ANOVA: CFA vs EAE $p < 0.001$; EAE vs mild-EAE $p < 0.001$). Finally, we tested the locomotor performance through the CatWalk gait analysis, which has been used especially in research of spinal cord injury (Hamers et al., 2006), but never to assess impaired gait function in EAE. By means of this automated analysis, we provided quantitative assessment of inter-limb coordination (regularity index), different static (base of support and stride length) and dynamic (duty cycle) gait parameters for individual paws. The degree of inter-limb coordination during the gait cycle, measured by the regulatory index, was significantly affected in EAE mice (63%), while it was almost 100% in fully motor coordinated animals, CFA and mild-EAE groups (Fig. 1D; CFA n = 11, EAE n = 5, mild-EAE n = 10; one-way ANOVA: CFA vs EAE $p < 0.001$; EAE vs mild-EAE $p < 0.001$). Regarding the paw analysis, the automated CatWalk system detected differences between CFA and EAE only for the hind prints (max contact area and print length), reflecting the less impairment of the front paws, as expected from the clinical score (Table 1). However, some parameters such as max contact, max intensity and stride length were significantly different for both the hind and the front paws between CFA and EAE (Table 1 and Fig. 1E), highlighting subtle abnormalities not detectable by the clinical scoring. Of note, mild-EAE mice showed paw statistics values similar to CFA group.

As an indicator of trunk stability of the animal, we measured the distance between the hind paws and between the front paws (BOS). As shown in Fig. 1F, only EAE group showed BOS values altered for both the hind and the front paws; the former were reduced and the latter increased (hind paws, one-way ANOVA: CFA vs EAE $p < 0.01$, EAE vs mild-EAE $p < 0.001$; front paws, one-way ANOVA: CFA vs EAE $p < 0.001$, EAE vs mild-EAE $p < 0.001$).

Duty cycle is the percent of the total step cycle during which a paw is on the ground. In EAE mice the time that the hind paws (LH and RH) were in contact with the floor was significantly reduced in comparison to CFA and mild-EAE mice, while the same parameter was found to be increased in the front paws (Fig. 1G; hind paws, one-way ANOVA: CFA vs EAE $p < 0.001$, EAE vs mild-EAE $p < 0.001$; front paws, one-way ANOVA: CFA vs EAE $p < 0.05$, EAE vs mild-EAE $p < 0.05$). These data mean that EAE hind paws fall to the ground slowly, as a result of a reduced stand and increased swing phase, while EAE front paws fall to the ground quickly, as consequence of increased stand and reduced swing phase. As observed for the other CatWalk parameters, mild-EAE behaved similarly to control CFA mice.

Overall, CatWalk results indicate that EAE mice show an instable gait, mainly caused by a pronounced disability of the hind paws and by an adaptation of the front paws. Of note, in mild-EAE we could not detect any signs of gait instability in terms of both static and dynamic parameters. Furthermore, the time mice spent to cross the walkway was analyzed in order to elucidate spontaneous voluntary activity: runs' duration resulted similar between CFA (1.762 ± 0.247 s) and mild-EAE mice (2.116 ± 0.089 s), while EAE mice spent more time to cross the runway (3.3 ± 0.253 s; CFA n = 11, EAE n = 5, mild-EAE n = 10; one-way ANOVA: CFA vs EAE $p < 0.001$; EAE vs mild-EAE $p < 0.01$; CFA vs mild-EAE $p > 0.05$, data not shown). In conclusion, all together the results obtained from rotarod, grip strength test and CatWalk, highlight the absence of motor impairment and motor fatigue in mild-EAE mice.

Based on this EAE classification, we performed the NB test, which assesses nesting ability that is a natural and instinctive behavior in rodents. Notably the NB ability was correlated to sickness behavior (Filali et al., 2009) and to psychiatric disorders mediated by inflammatory activity (Takao et al., 2013). We observed that both 20 dpi EAE groups had significantly reduced nesting scores compared to controls (Figs. 1H–H′; n = 7 for all groups; non parametric ANOVA Kruskal–Wallis test: CFA vs EAE $p < 0.05$; CFA vs mild-EAE $p < 0.01$). Interestingly, nesting performance of the two EAE groups was very similar despite their different degree of motor disability. This result strongly suggested that EAE causes a worse social interaction and less motivation for daily living activity independently of motor deficits in the acute phase of the disease.

Starting from these results, we decided to perform TST and FST only on mice without motor impairment (mild-EAE) since these are very demanding tests and require a regular tail tone. As highlighted by the FST in Fig. 1I, 21 dpi mild-EAE mice showed increased immobility compared to CFA mice (n = 7 per group; unpaired Student's *T*-test: $p < 0.05$). The performance of 22 dpi mild-EAE mice at TST compared to CFA mice confirmed the depressive-like behavior observed at FST (Fig. 1J; unpaired Student's *T*-test: $p < 0.05$, n = 7 per group), suggesting increased depressive phenotype in mild-EAE mice. Moreover, to further exclude any effect caused by motor fatigue on FST and TST, we performed the same tests during the preclinical phase of the disease (before the onset of motor deficit, 9 dpi). Accordingly, EAE mice compared to CFA

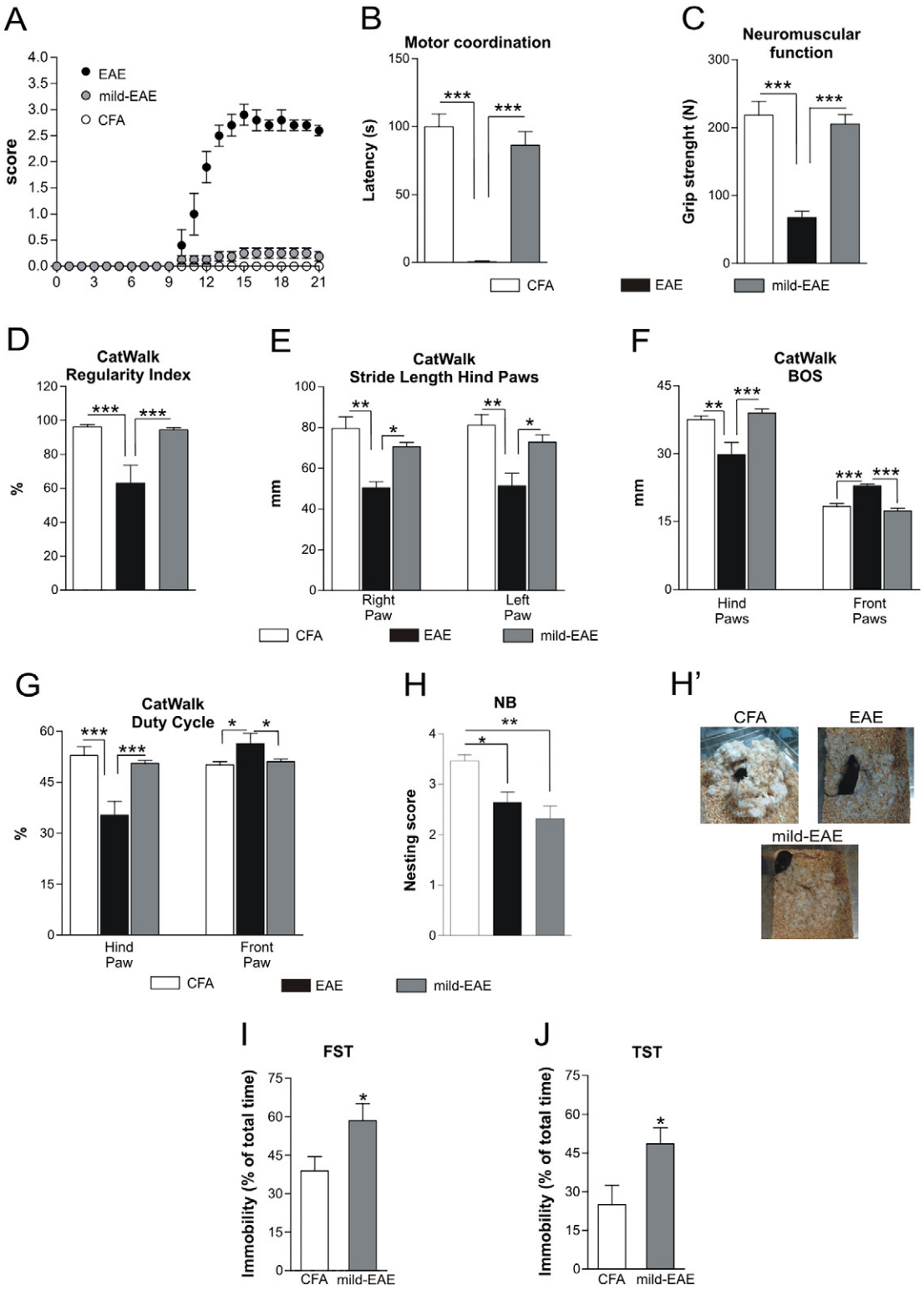

Fig. 1. EAE mice develop mood disorders independently of motor deficit. Motor assessment was performed on EAE mice at the peak of the acute phase of the disease (20 dpi). A. Clinical score of EAE and CFA mice. EAE mice were divided into two groups on the basis of maximum clinical deficit developed during the course of the disease: score < 1 for mild-EAE and score ≥ 1 for EAE. Data were obtained by pulling together three different immunizations. Numbers on the horizontal axis are days post-immunization (dpi). B. Rotarod performance expressed as time (seconds) to fall off the rod (latency) of mild-EAE was similar to control CFA mice, while EAE mice fell off immediately as the rod began rotating, as a consequence of highly compromised motor coordination. C. Cumulative fore- and hind-limb grip strength analysis revealed no differences between CFA and mild-EAE mice and the impairment of the neuromuscular function in EAE mice. D–G. Gait parameters assessed by CatWalk analysis showed that mild-EAE mice had preserved over ground locomotion. D. Inter-limb coordination, measured by the regularity index, was impaired in EAE mice and almost normal in mild-EAE mice. The static parameters stride length (E) and BOS (F) of the hind paws were significantly reduced in EAE mice compared to both CFA and mild-EAE mice, as well as the dynamic parameter duty cycle (G). Conversely, both BOS and duty cycle were significantly increased in the front paws of EAE mice (F, G). H. Nest building was performed on EAE, mild-EAE and CFA mice at 20 dpi. The nest building ability of EAE and mild-EAE mice is significantly impaired compared to CFA mice. H′. Representative photographs of nest built by CFA, EAE and mild-EAE mice. I–J. Depressive-like behavior was investigated only in mild-EAE (score < 1). Bar graphs in I and J represent the immobility at FST (21 dpi) and TST (22 dpi) respectively of mild-EAE vs CFA. The statistical differences for three group comparisons were analyzed by one-way ANOVA followed by Tukey's HSD, except for nesting score for which the non-parametric Kruskal–Wallis test followed by Dunn's comparisons was applied; Unpaired *T*-test was used for two-group analysis. Values are means ± SEM, ***p < 0.001, **p < 0.01, *p < 0.05.

Table 1
Quantitative automated CatWalk analysis of 20 dpi EAE, mild-EAE and CFA mice.

CatWalk parameters	CFA	EAE	Mild-EAE
Print length RF (mm)	8.617 ± 0.362	7.819 ± 0.960	8.348 ± 0.350
Print length LF (mm)	8.944 ± 0.386	7.210 ± 1.002	8.209 ± 0.208
Print length RH (mm)	8.610 ± 0.325	6.998 ± 1.590	8.482 ± 0.125
Print length LH (mm)	8.642 ± 0.213	4.025 ± 0.599***	8.317 ± 0.262###
Max contact area RF (mm^2)	27.290 ± 2.504	23.745 ± 6.029	26.601 ± 3.080
Max contact area LF (mm^2)	26.464 ± 2.497	21.139 ± 5.158	24.245 ± 2.525
Max contact area RH (mm^2)	26.035 ± 1.750	12.988 ± 3.636***	23.759 ± 1.147##
Max contact area LH (mm^2)	24.091 ± 1.689	6.218 ± 0.705***	20.766 ± 1.413###
Stride length RF (mm)	82.700 ± 5.539	51.545 ± 1.495***	75.636 ± 2.404##
Stride length LF (mm)	82.000 ± 5.244	50.590 ± 3.384***	76.140 ± 2.753##
Max contact max intensity RF	188.802 ± 2.033	159.940 ± 16.363**	191.341 ± 2.315##
Max contact max intensity LF	191.672 ± 1.745	157.631 ± 18.166**	191.254 ± 2.003##
Max contact max intensity RH	203.609 ± 0.913	130.563 ± 16.099***	201.204 ± 1.974###
Max contact max intensity LH	113.608 ± 3.246	70.869 ± 1.153***	115.421 ± 3.778###

Values are mean ± sem. Statistical analysis: EAE vs CFA **$p < 0.01$, ***$p < 0.001$; mild-EAE vs CFA ##$p < 0.01$, ###$p < 0.001$

mice were significantly different in terms of immobility in both tests (Fig. 1S A, B; n = 9 for all groups; unpaired Student's *T*-test: CFA vs EAE $p < 0.05$ for both tests). These results strongly suggested that EAE causes depressive-like behaviors independently of motor deficits.

IL-1β is involved in mood alterations in EAE mice

IL-1β is one of the main pro-inflammatory cytokines responsible for behavioral alterations (Dantzer et al., 2008). Notably, IL-1β expression is increased in EAE (Mandolesi et al., 2013) and MS brains (Rossi et al., 2012). Therefore, we focused our attention on the putative role of IL-1β in goal-directed behavior in EAE mice. First, we assessed the expression of IL-1β in three different brain areas, involved in mood control, such as the striatum, the hippocampus and the amygdala (Dantzer et al., 2008). We previously observed that besides a strong microgliosis, EAE hippocampus, striatum and cerebellum showed a prominent astroglial activation and CD3+ lymphocyte infiltration as potential sources of IL-1β (Grasselli et al., 2013; Mandolesi et al., 2013; Mori et al., 2014). Since microglia is the main source of cytokines during the course of neuroinflammation (Mandolesi et al., 2013; Nisticò et al., 2013), we performed double immunofluorescence for IL-1β and Iba-1, marker of microglia.

We found that EAE group showed high expression levels of IL-1β (red staining) in the presence of prominent microglia activation (Iba-1 staining in green) in comparison with CFA group in both the striatum (Fig. 2A) and the hippocampus (Fig. 2B). Conversely, the extent of microgliosis was less marked in the amygdala of EAE mice and IL-1β labeling could not be detected (Fig. 2C). In accordance with the immunohistochemistry, qRT-PCR experiments revealed a significant induction of IL-1β mRNA in both the striatum (CFA n = 9, EAE n = 7; unpaired Student's *T*-test: $p < 0.01$) and hippocampus (CFA n = 5, EAE n = 5; unpaired Student's *T*-test: $p < 0.05$) of EAE mice. Conversely, we could not detect significant differences between EAE and CFA amygdala (CFA n = 5, EAE n = 7; unpaired Student's *T*-test: $p > 0.05$), although a recent study described increased IL-1β mRNA in such area (Acharjee et al., 2013) (Fig. 2D). Taken together data from immunohistochemistry and q-RT-PCR suggested that IL-1β might modulate behavioral responses during the course of EAE, by acting mainly on the striatum and the hippocampus.

To further search a link between emotional brain area, inflammation and depressive-like behavior we next assessed the expression of IL-1β in the striatum and the hippocampus of mild-EAE mice. The expression of IL-1β was still present in both structures, in a context of mild microglia activation (Figs. 3A, B; IL-1β red staining, Iba-1 green staining). Arrows in the figures indicate the presence of lesions in both tissues, where there are IL-1β positive microglial cells. By qRT-PCR we could detect significant levels of IL-1β expression only in the striatum of mild-EAE mice compared to their relative controls (Fig. 3C; striatum: CFA n = 7, EAE n = 6; unpaired Student's *T*-test: $p < 0.05$; hippocampus: CFA n = 8, EAE n = 11; unpaired Student's *T*-test: $p > 0.05$). Interestingly, the expression of TNF-α, another important cytokine involved in mood abnormalities (Haji et al., 2012), was not changed in both the striatum (CFA, n = 7 mRNA fold change: 1.11 ± 0.2; EAE, n = 6 mRNA fold change: 1.26 ± 0.37; unpaired Student's *T*-test: $p > 0.05$) and the hippocampus (CFA, n = 6 mRNA fold change: 1.02 ± 0.079; EAE n = 8 mRNA fold change: 0.77 ± 0.200; unpaired Student's *T*-test: $p > 0.05$) of mild-EAE mice compared to CFA (not shown).

Altogether these experiments might correlate the presence of IL-1β in the striatum of EAE mice with their depressive-like behavior, suggesting that even a little amount of IL-1β, could induce mood alterations in these mice.

Central blockade of IL-1β corrects mood disturbances of EAE mice

To investigate the role of IL-1β in EAE mood abnormalities, we next performed a chronic inhibition of the IL-1β signaling in EAE mice by icv delivery of IL-1ra and we analyzed behavior during the acute phase of the disease. As previously shown, this preventive treatment reduced motor disability in EAE mice and the inflammatory reaction in acute phase EAE cerebellum and hippocampus (Mandolesi et al., 2013; Mori et al., 2014). Accordingly, here we observed a milder microglia activation in the striatum of EAE-IL-1ra treated mice relative to EAE-vehicle group (Fig. 4A).

As for experiments in Fig. 1, FST and TST tests were performed only on EAE animals without motor dysfunctions at 21 and 22 dpi respectively. IL-1ra corrected the behavioral alterations induced by EAE since the immobility of EAE at FST was similar to CFA-vehicle group (Fig. 4B: CFA-vehicle n = 7; EAE-vehicle n = 7; EAE-IL-1ra n = 8; one-way ANOVA: CFA-vehicle vs EAE-vehicle $p < 0.05$; EAE-vehicle vs EAE-IL-1ra $p < 0.05$). However, we could not appreciate the beneficial effect of IL1-ra treatment observed in FST when mice were exposed to TST paradigm (CFA-vehicle n = 7; EAE-vehicle n = 7; EAE-IL-1ra n = 8; Fig. 4C; $p > 0.05$ for all comparisons, one-way ANOVA analysis p = 0.053), probably due to a different sensitivity of the two tests in revealing the anti-depressive property of IL-1ra treatment (Cryan et al., 2005).

Altogether, our results suggest that IL-1ra treatment can improve not only motor symptoms but also EAE-associated mood disturbances through IL-1β signaling inhibition.

Defective dopaminergic neurotransmission in striatum of EAE mice

We hypothesized that the depressive symptoms of EAE mice could be mediated, at least in part, by altered dopaminergic transmission, since pharmacological blockade of DA receptors is known to alter TST

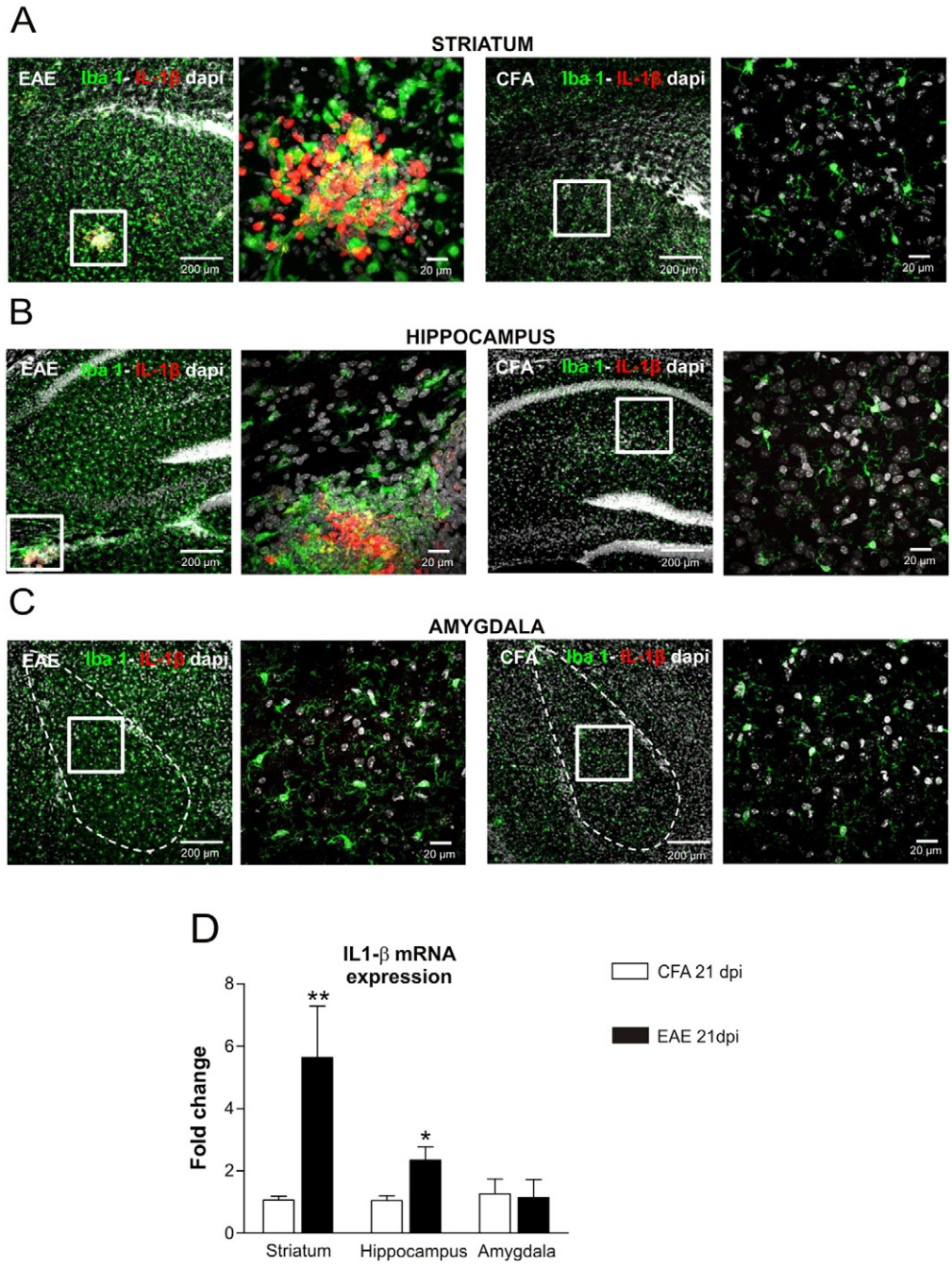

Fig. 2. IL-1β is up-regulated in the striatum and hippocampus of EAE mice. A–C. Double immunostaining of brain coronal sections (in gray DAPI nuclei) showing expression of Iba1-positive microglia/macrophage cells (green) and of IL-1β (red) in EAE and CFA mice (21 dpi). A, B. In both the striatum (A) and the hippocampus (B), Iba1 and IL-1β expression were strongly up-regulated in EAE mice (left panels) in comparison with their relative CFA groups (A, B right panels). The white boxes in each panel represent area selected for acquisition at high magnification and showed on the right; IL-1β was abundantly expressed in active inflammatory lesions in EAE groups and in the presence of strong microglia activation. C. Conversely, both inflammatory lesions and IL-1β staining were undetectable in the basolateral amygdala of EAE mice. Accordingly, a less prominent microglia activation seemed to characterize the amygdala of EAE mice in comparison with the other brain areas. Scale bars in EAE and CFA panels: 200 μm in the left and 20 μm in the right. D. The levels of IL-1β mRNA were quantified in the striatum, hippocampus and amygdala by qRT-PCR, using β-actin as internal control. No statistical differences were detectable in the amygdala between CFA and EAE mice during the acute phase of the disease (21 dpi). Conversely, in both striatum and hippocampus of EAE mice an up-regulation of the mRNA levels was evident. Statistical test used for comparison was the unpaired *T*-test. Values are means ± SEM, $^{**}p < 0.01$, $^{*}p < 0.05$, versus CFA control mice.

and FST performance in mice (David et al., 2003; Ripoll et al., 2003) and nest building abilities are impaired in MPTP-induced dopamine deficiency (Sager et al., 2010).

We measured therefore DA release in striatal brain slices of EAE (20–25 dpi) and control mice through CPA experiments. Measurements from EAE (number of animals = 5) and mild-EAE (number of animals = 4) were pooled together because their values were similar (unpaired Student's *T*-test: $p > 0.05$), supporting the hypothesis that the mechanisms related to mood disturbances were independent from motor deficits.

In response to an increasing stimulation protocol, at the maximal stimulation intensity (1000 μA; 20–40 μs) a plateau of DA release was reached. The maximum striatal extracellular DA release evoked in the EAE group was significantly lower (n = 27 EAE and n = 23 mild EAE)

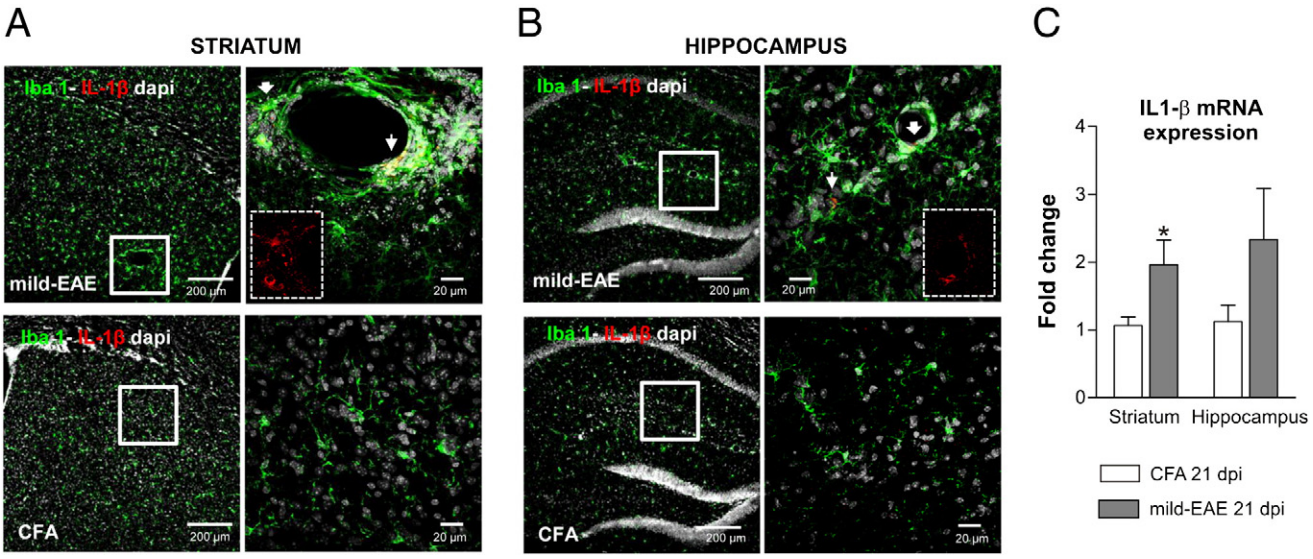

Fig. 3. IL-1β is up-regulated in the striatum of mild-EAE. A, B. Double immunostaining of brain coronal sections (in gray DAPI nuclei) showing expression of Iba1-positive microglia/macrophage cells (green) and of IL-1β (red) in mild-EAE and CFA mice (21 dpi). In both striatum (A) and hippocampus (B), a more pronounced expression of Iba1 and IL-1β was clearly detectable in mild-EAE mice (upper panels) in comparison with their relative CFA groups (A, B bottom panels). The white boxes in each panel (A, B) represent areas selected for acquisition at high magnification and showed on the right; although the inflammatory reaction in these mice was less prominent relative to EAE groups, IL-1β was still detectable in both brain areas in the presence of a moderate microglia activation (arrowheads). The dashed white boxes show IL-1β staining in the area indicated by the big arrows. Scale bars in A and B: 200 μm in the left and 20 μm in right. C. Histogram showed the IL-1β mRNA fold change (±SEM), detected by qRT-PCR, in the striatum and the hippocampus of mild-EAE versus CFA mice. β-actin was used as internal control. Statistical significance was evaluated by unpaired *T*-test: *$p < 0.05$.

compared with that evoked in the CFA group (number of animals: 5; n = 35; unpaired Student's *T*-test: $p < 0.001$) (Figs. 5A, A′). Furthermore, we examined the pharmacological induction of DA release produced by the perfusion of amphetamine (30 μM, ~10 min) on corticostriatal slices, to explore a possible deficit of DA vesicle content. The amperometric data revealed no significant difference between CFA (n = 10) and EAE mice (n = 4 EAE and n = 6 mild EAE) under forced DA synaptic release (unpaired Student's *T*-test: $p > 0.05$) (Fig. 5B).

We further investigated the effects of EAE on striatal DA system, by WB measurements of a number of pre- and postsynaptic parameters of DA transmission. Biochemical results here shown are representative of WB in which mild-EAE mice were compared to relative controls: the same results were obtained comparing EAE to CFA in different blots. EAE failed to alter tyrosine hydroxylase (TH) expression, the enzyme rate limiting in the synthesis of DA (unpaired Student's *T*-test: $p > 0.05$; n = 4 each group) (Figs. 5C, C′). The levels of both DA D1- and D2-like receptors (D1R and D2R), the main DA receptors expressed within the striatum, were unchanged in total striatal extracts (unpaired Student's *T*-test: $p > 0.05$ for both comparisons; n = 4 each group) (Figs. 5C, C″, C‴). The expression levels of the DA- and cAMP-regulated phosphoprotein 32 kDa (DARPP32), found in a great majority of the MSNs, were unaffected by EAE (unpaired Student's *T*-test: $p > 0.05$; n = 4 each group) (Figs. 5C, C″).

Due to the strong effect exerted by IL1-ra treatment on EAE depressive-like behavior, we next examined the effects of icv IL1-ra infusion on the evoked DA outflow in EAE mice (n = 10). There was not a significant difference (unpaired Student's *T*-test: $p > 0.05$) between EAE-IL1-ra and CFA control group, thus showing a complete rescue in the release of DA in treated mice (Figs. 6A, A′).

To better understand the biochemical basis of DA release modulation in EAE striatum, we evaluated the phosphorylation state of DARPP32. Indeed, DARPP32 undergoes phosphorylation mainly on threonine 34 (Th34) and threonine 75 (Th75) and DA regulates the state of phosphorylation of DARPP32 at these sites in a bidirectional manner (Svenningsson et al., 2004). In total striatal extracts the phosphorylation at Th75 was unchanged between EAE-vehicle (n = 4) and CFA-vehicle (n = 4) and therefore not modulated by IL-1ra treatment (n = 5; one-way ANOVA: $p > 0.05$ for all comparisons) (Figs. 6B, B′). Conversely, the phosphorylation on Th34 site was up-regulated in EAE extracts and entirely recovered by IL-1ra treatment (one-way ANOVA: CFA-vehicle vs EAE-vehicle $p < 0.05$; EAE-vehicle vs EAE-IL-1ra $p < 0.001$) (Figs. 6C, C′), without changes in the expression of total DARPP32 among groups (one-way ANOVA: $p > 0.05$ for all comparisons) (Figs. 6C, C″). Th34-DARPP32 phosphorylation is PKA-dependent upon D1 and adenosine A2A receptors (A2AR) stimulation: A2ARs negatively control D2Rs activity (Svenningsson et al., 2004). Considering the observed reduction of DA release in EAE striatum, we hypothesize that such over-phosphorylation at Th34 site was likely due to over-sensitization of D1R and reduced signaling through D2R.

Discussion

The present study provides first evidence of the link between depressive-like behavior and alteration of striatal dopaminergic transmission in EAE mice. We showed the independence of mood changes in EAE mice from motor disability, demonstrating the occurrence of depressive-like behavior in acute phase EAE mice without motor impairment. Moreover, we linked EAE behavioral phenotype to increased expression of IL-1β in the striatum and to altered dopaminergic transmission in the striatum of acute EAE. Finally, we found that central blockade of IL-1β signaling reversed both mood and striatal DA neurotransmission alterations associated with EAE.

Depression is often diagnosed in MS patients (Feinstein, 2007) even in the absence of neurological disability (Suh et al., 2010), suggesting a difference between an “intrinsic” depression from a “reactive” one. In this respect, despite the limitations of behavioral studies in rodents, research on the EAE model can highlight the pathophysiology of mood alterations in MS. Previous observations revealed both anxious- and depressive-like behaviors in association with brain inflammation (Acharjee et al., 2013; Haji et al., 2012) in a very precocious phase of the disease, before the appearance of motor deficits. On the other hand, the characterization of a depressive-like behavior in the presence of motor disability during the acute phase of the disease has always

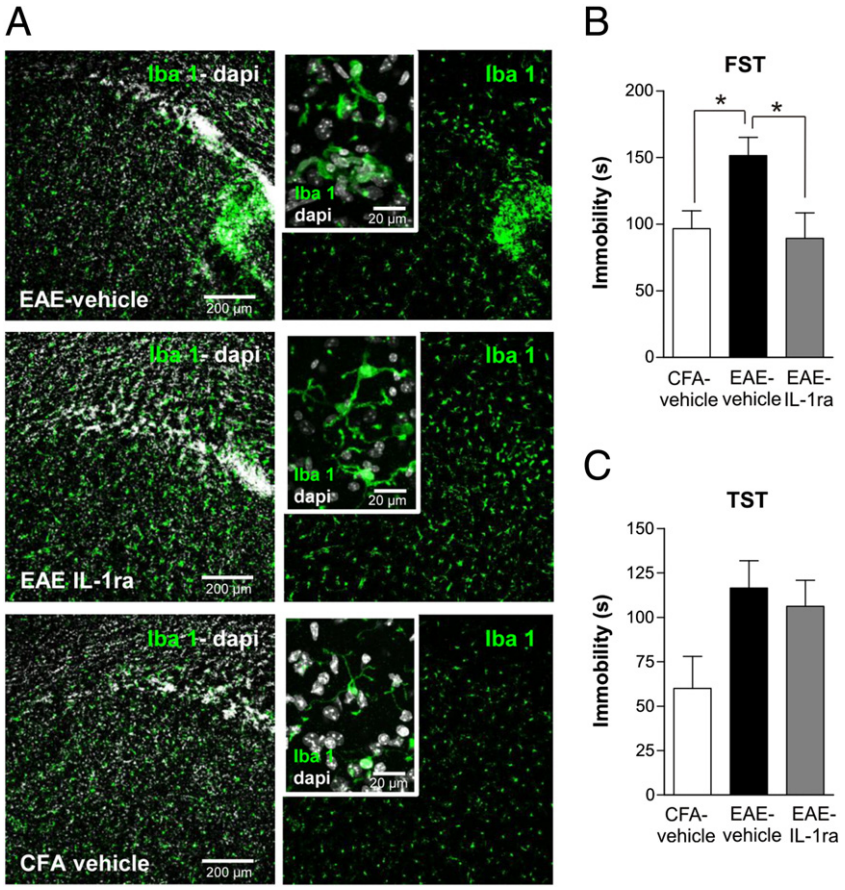

Fig. 4. Central blockade of IL-1β corrects emotional disturbances of EAE mice. A. Immunostaining of cortico-striatal sections showing expression of Iba1-positive microglia/macrophage cells (green) and DAPI staining of the cell nuclei (gray). In EAE-IL-1ra slices (central panels) microglia activation was less prominent compared with EAE mice (upper panels) but more pronounced relative to CFA mice (bottom panels). The insets are high magnifications showing the morphology of Iba-1 positive cells, which seem mildly activated in EAE-IL-1ra striatum. Scale bars: 200 μm for low magnification images and in 20 μm for high magnification images. B, C. Depressive-like behavior was investigated in acute phase of the disease in mild-EAE mice: the histogram in B shows the reduction of immobility of 21 dpi EAE-IL-1ra mice in comparison to vehicle group at FST. As shown by the bar graph in C the performance of EAE-IL-1ra at TST did not replicate the recovery of immobility observed at FST (22 dpi). Values are means ± SEM. Statistical differences were analyzed by one-way ANOVA followed by Tukey's HSD. *$p < 0.05$.

been a challenging issue. Former study by Pollak and colleagues showed sickness behavior in EAE mice during the acute phase of the disease by performing behavioral tests (sucrose preference and food intake tests) that are not influenced by motor disability (Pollak et al., 2000). In the present work, by taking advantages of mice with mild EAE phenotype (mild-EAE), which represent the 20% of MOG-immunized animals in our experimental conditions, we could address a role for inflammation in mood alterations during the acute phase of the disease and dissect in part the cellular and molecular mechanisms involved. First, in order to overcome limitations linked to testing behavior in mice with motor disabilities, we characterized the motor skills of mice with mild EAE phenotype at the peak of the acute phase of the disease (20–22 dpi) in comparison to EAE and CFA mice. In this regard, it is noteworthy the novel and complete picture of severely diseased EAE mice gait depicted by CatWalk analysis. Data from CatWalk highlighted a typical EAE gait, characterized by increased double-limb support in the front paws, as a consequence of trunk instability, due to strong impairment of the hind paws. Such posture seems to be specific to EAE, if compared to gait observed in rats after spinal cord injury (Hamers et al., 2006; Garcia-Ovejero et al., 2014), and likely due to inflammatory demyelination occurring in EAE. By means of well-established tests for evaluating motor fatigue such as grip test and rotarod (Murphy et al., 2012; Norden et al., 2014), we verified that mild-EAE mice showed preserved coordination and neuromuscular functions. Also, the detailed CatWalk gait analysis confirmed normal gait in these mice. Despite of the lack of motor disability, mild-EAE mice showed the same NB skills of motor impaired EAE mice. The NB test, which is not stressful and driven by a natural predisposition, has been used to test changes in emotional states, such as apathy and social withdrawal. Nest building abilities in mice can be interpreted as a measure of their interests in surroundings and motivation (Aubert, 1999) and can be perturbed in consequence of cortical pathology (Boissonneault et al., 2009) and dopamine deficiency (Sager et al., 2010). Considering that the cortex undergoes atrophy and inflammatory reaction in EAE (Mackenzie-Graham et al., 2012), a contribution of cortical deficits to reduced nest abilities cannot be fully excluded. Of note, the NB test has been proven a good tool in revealing social behavior alterations in another autoimmune model (Blossom et al., 2008). Here, the NB test allowed not only to reveal motivation-based behavior changes in severely impaired EAE mice, but also to unmask such behavioral disturbances in mild-EAE mice during the acute phase of the disease. Furthermore, immobility in both FST and TST increased in mild-EAE mice, suggesting reduced active coping response to stress, which has been linked to depressive-like behavior (Cryan et al., 2005; Petit-Demouliere et al., 2005). Nevertheless, immobility in FST and the other behavioral changes observed in the present study could be secondary to other mechanisms. In this respect, other tests, like sucrose preference and social interaction, could be useful to examine in depth EAE behavioral phenotype. In addition, to exclude the contribution of motor deficits on behavioral tests, FST and TST were performed before the onset of clinical symptoms (preclinical phase),

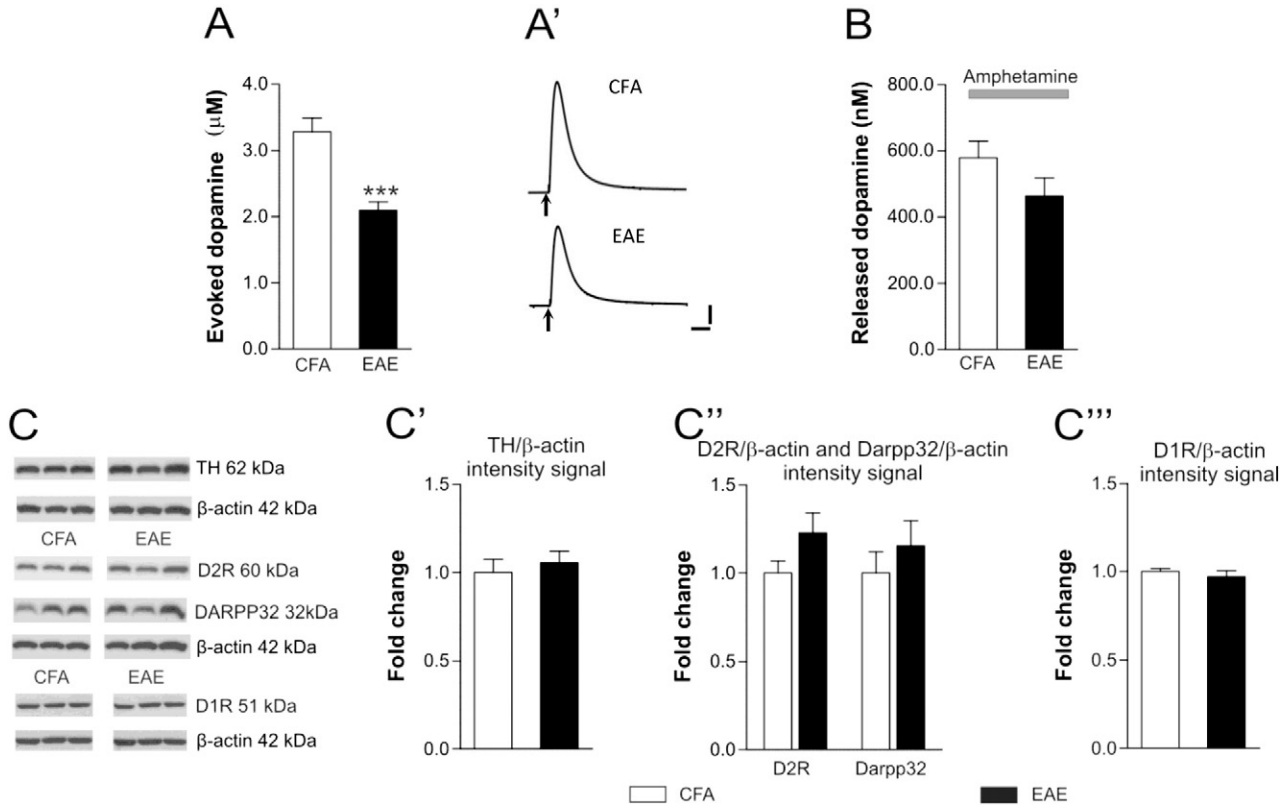

Fig. 5. Dopaminergic neurotransmission is altered in EAE striatum. A. Amperometric measurement of stimulus- and amphetamine-evoked changes of striatal extracellular DA levels (expressed in μM) in CFA and EAE mice. The graph illustrates striatal extracellular DA levels in response to maximal stimulation (1 mA/ 40 μs). Note that in EAE mice, the DA release is significantly lower than that in CFA mice. A'. Representative amperometric signals recorded in the dorsal striatum in brain slices obtained from CFA and EAE mice showing different amplitude at maximal stimulation. Vertical bar indicates 50 mV (amplitude), horizontal bar indicates 500 ms (duration); the arrow indicates stimulation start point. B. The graph shows the amphetamine-induced DA overflow (expressed in nM) in CFA and EAE mice. Note that the difference between the two groups of mice is not statistically significant. C–C‴. Biochemical study of the main dopaminergic markers was carried out on striatal protein extracts from acute phase EAE and CFA groups. The panels in C show representative blots comparing the two experimental groups, for each protein detected. WB analysis revealed no substantial changes in the expression of TH (C'), D2R (C″), D1R (C‴) and DARPP32 (C″) relative to β-actin between EAE and CFA striata. WB data are normalized to CFA values. Values are means ± SEM. Unpaired *T*-test was used for two-group analysis. Values are means ± SEM, ***$p < 0.001$.

highlighting a depressive-like behavior independent of motor impairment. These results support previous observations made in a suboptimal immunization model of EAE (Peruga et al., 2011). Altogether these results strongly corroborate the hypothesis of the presence of 'intrinsic' mood disturbances in acute EAE mice which are independent from motor impairments, as likely occurs in MS.

IL-1β and TNF-α have been convincingly associated with mood disorders (Dantzer et al., 2008) and their levels have been found higher in serum and CSF of MS patients (Imitola et al., 2005), and in EAE brains (Haji et al., 2012; Mandolesi et al., 2013). Of note icv injection of IL-1β causes sickness behavior, anxiety and depression in mice (Bluthé et al., 1995; Koo and Duman, 2008). Here, we linked EAE behavioral syndrome with inflammation in the striatum, brain area involved in both motor and mood regulation in humans and rodents (Báez-Mendoza and Schultz, 2013; Chesselet and Delfs, 1996) and affected by EAE (Centonze et al., 2009). We found that IL-1β was selectively increased with respect to TNF-α in the striatum of mild-EAE mice, together with reduced microglia activation. Hippocampus of mild-EAE brains showed variable and not significant levels of both IL-1β and TNF-α expression. Although we cannot exclude a role for IL-1β expressed in the hippocampus in inducing EAE depressive-like behavior, our data suggest a major role of IL-1β in the striatum. Additionally, we corrected EAE behavioral abnormalities by blocking IL-1β signaling through central delivery of IL-1ra, thus indicating the involvement of IL-1β in EAE-linked depression. In this respect, a previous report showed the effectiveness of IL-1ra peripheral treatment in attenuating EAE-associated decrease in food and sucrose intake (Pollak et al., 2003).

Many symptoms of depression are consistent with dysfunction of the basal ganglia and of the DA system (Felger and Miller, 2012), in conjunction with detrimental effects of cytokines (Zhu et al., 2006). Notably, changes in striatal connectivity have been claimed to be predictive for episode relapse risk in major depression (Meng et al., 2014). Also, evidence exists for the involvement of the dopaminergic system in both motor and behavioral outcomes in MS and EAE.

In MS, the "dopaminergic machinery" of lymphocytes (Cosentino et al., 2002), and also DA neuronal metabolism (Markianos et al., 2009) are compromised, a finding that is in line with reduced monoamine concentrations in EAE brain and spinal cord (Musgrave et al., 2011a, 2011b). Indeed, not only dopamine but also serotonin and norepinephrine concentrations are reduced in EAE brains, and increasing their levels by using the MAO inhibitor phenelzine improves both motor and behavioral outcomes of EAE mice (Benson et al., 2013). Notably, D1R antagonists but not D2R antagonists ameliorate the clinical score of PLP-immunized SJL mice (Nakano et al., 2008).

Since the role of serotoninergic and noradrenergic systems in rodents behavior is well established (Krishnan and Nestler, 2008), we cannot exclude a contribution of these neurotransmitters to EAE depressive-like behavior. However, in the present study we uncovered an impairment of the dopaminergic system in the striatum of EAE mice still unknown, and we correlated it to EAE behavioral disorders (both motivational and depressive-like behavior) and striatal expression of IL-1β. CPA measurements revealed a reduced stimulus-induced DA outflow in EAE mice not due to a deficit of DA content in the synaptic terminals. It might be possible that the activation of

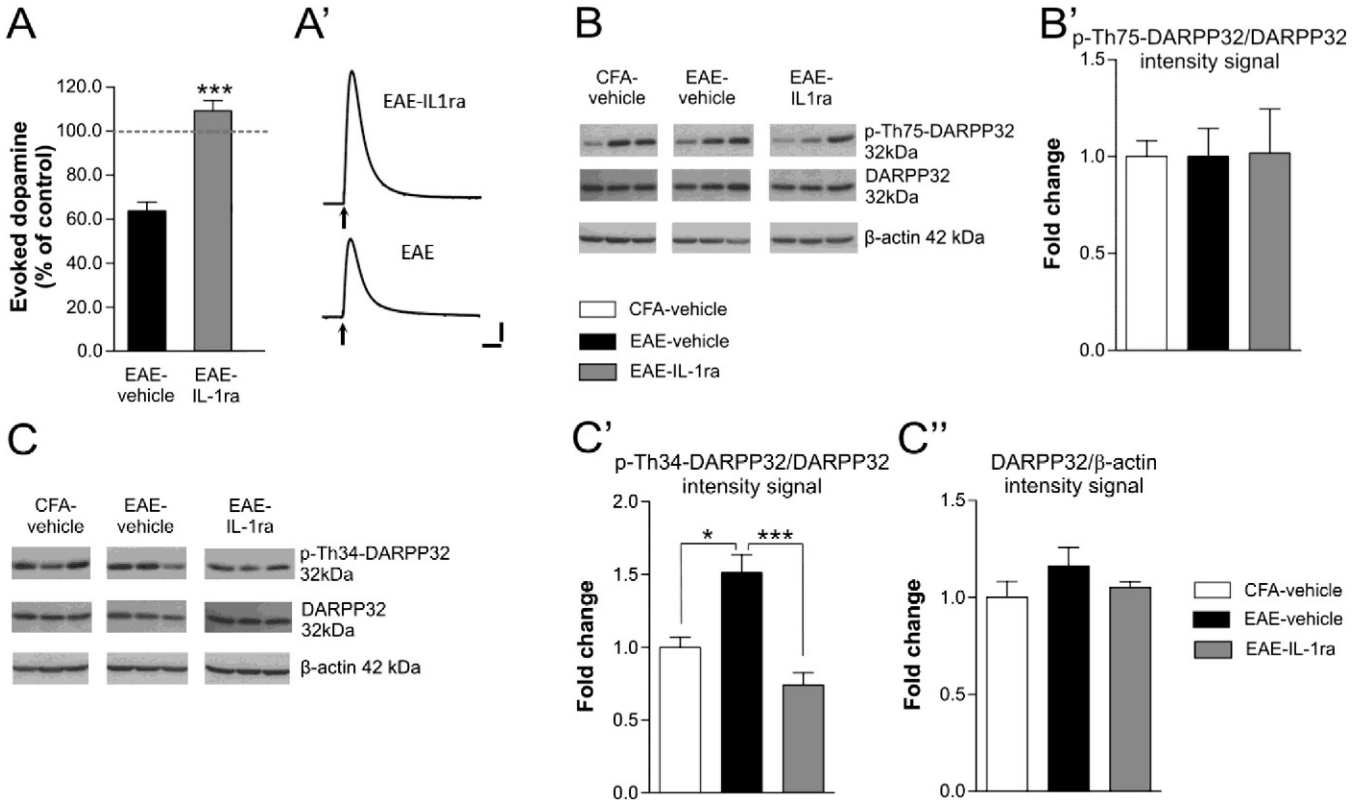

Fig. 6. IL-1ra icv treatment recovers defective dopaminergic neurotransmission in EAE striatum. A. Normalized amperometric measurements of the stimulus-evoked striatal DA outflow in EAE-IL-1ra mice (109.14 ± 4.69%). Note the non-significant increase of DA release respect to CFA control, but the significant increase in release respect to EAE mice (63.71 ± 3.96%). A′. Representative traces showing DA release in EAE-IL-1ra mice and EAE mice. Vertical bar indicates 50 mV (amplitude), horizontal bar indicates 500 ms (duration); the arrow indicates stimulation start point. B. WB performed on striatal homogenates from acute CFA-vehicle, EAE-vehicle and EAE-IL-1ra mice depicts the bands relative to p-Th75-DARPP32, DARPP32 and β-actin. The quantitative analysis reported in the graph B′ shows no significant changes among groups in the phosphorylation status of DARPP32 at Th75 residue. C. Western blot panel showing phosphorylation status of DARPP32 at Th34 site, the total substrate DARPP32 and β-actin. The bar graph in C′ reveals increased expression of p-Th34-DARPP32 relative to total DARPP32 content in EAE-vehicle compared to CFA-vehicle striata: IL-1ra in vivo treatment caused full rescue of the phosphorylation extent at Th34 of DARPP32. Neither EAE nor IL-1ra treatment affected the basal level of DARPP32, as illustrated in the histogram in C″. WB data are normalized to CFA values. Statistical differences were analyzed by one-way ANOVA followed by Tukey's HSD. ***$p < 0.001$, *$p < 0.05$.

synaptic machinery consequent to membrane depolarization is altered in EAE mice to reduce DA release, while the DA overflow caused by amphetamine does not require the same mechanism of action (Scarponi et al., 1999). By WB analysis of striatal total extracts we could not detect major differences in the expression of TH between EAE and CFA, indicating no changes in the synthesis of DA and corroborating the amperometric results. Neither D1R nor D2R levels were affected by EAE. Nevertheless, we observed an increase in the phosphorylation at Th34 of DARPP32, a well-studied molecular target for the actions of DA and Adenosine systems and highly enriched in virtually all MSNs in the striatum (Surmeier et al., 2007). Activation of D1Rs and/or A2ARs, via stimulation of PKA, leads to phosphorylation of DARPP32 at Thr34: A2ARs in turn negatively control D2R (Svenningsson et al., 2004). Since DA release was reduced in EAE striatum, we concluded that stimulation through D2R was reduced in these mice, leading to potentiation of D1- and/or A2A–PKA pathway. Interestingly, D1R–D2R unbalanced signaling may account for the EAE striatal glutamatergic potentiation (Centonze et al., 2009; Surmeier et al., 2007). Finally, IL-1ra treatment was able to increase DA striatal levels in EAE striatum and to restore normal phosphorylation levels of Th34-DARPP32, supporting the hypothesis of IL-1β-dependency of dopaminergic system alteration.

In conclusion, our data depict a scenario in which IL-1β impairs the dopaminergic signaling in the striatum, contributing to alter mood in EAE mice. Moreover, they clearly demonstrate that mood alterations are independent of motor disability in this neuroinflammatory condition, thus underlying the importance of screening and treating also MS patients without disability for anxious and depressive symptoms.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.11.022.

Funding and disclosure

This investigation was supported by the Italian National Ministero della Università (Grant No. 2010BN3MXM_007) to DC, by the Italian National Ministero della Salute (Grant No. GR11.148) to AM; by a grant from the European Community (Grant No. 21 4003) (AXREGEN: Axonal regeneration, plasticity & stem cells — Grant agreement 21 4003) and a founding PhD fellowship to NH. DF is supported by a Fondazione Italiana Sclerosi Multipla (FISM) fellowship (No. 2012/B/3). AU is a recipient of NARSAD Independent Investigator Grant 2013 from the Brain & Behavior Research Foundation (Grant No: 20353) and represents Mariano Scippacercola Foundation.

The authors declare no competing financial interests.

Acknowledgments

The authors thank Vladimiro Batocchi and Massimo Tolu for excellent technical assistance.

References

Acharjee, S., Nayani, N., Tsutsui, M., Hill, M.N., Ousman, S.S., Pittman, Q.J., 2013. Altered cognitive–emotional behavior in early experimental autoimmune encephalitis-cytokine and hormonal correlates. Brain Behav. Immun. 33, 164–172.

Aktas, O., Zipp, F., 2003. Regulation of self-reactive T cells by human immunoglobulins—implications for multiple sclerosis therapy. Curr. Pharm. Des. 9, 245–256.

Aubert, A., 1999. Sickness and behaviour in animals: a motivational perspective. Neurosci. Biobehav. Rev. 23, 1029–1036.

Báez-Mendoza, R., Schultz, W., 2013. The role of the striatum in social behavior. Front. Neurosci. 7, 233.

Benson, C.A., Wong, G., Tenorio, G., Baker, G.B., Kerr, B.J., 2013. The MAO inhibitor phenelzine can improve functional outcomes in mice with established clinical signs in experimental autoimmune encephalomyelitis EAE. Behav. Brain Res. 252, 302–311.

Bermel, R.A., Innus, M.D., Tjoa, C.W., Bakshi, R., 2003. Selective caudate atrophy in multiple sclerosis: a 3D MRI parcellation study. Neuroreport 14, 335–339.

Blossom, S.J., Doss, J.C., Hennings, L.J., Jernigan, S., Melnyk, S., James, S.J., 2008. Developmental exposure to trichloroethylene promotes CD4+ T cell differentiation and hyperactivity in association with oxidative stress and neurobehavioral deficits in MRL+/+ mice. Toxicol. Appl. Pharmacol. 231, 34–53.

Bluthé, R.M., Beaudu, C., Kelley, K.W., Dantzer, R., 1995. Differential effects of IL-1ra on sickness behavior and weight loss induced by IL-1 in rats. Brain Res. 677, 171–176.

Boissonneault, V., Filali, M., Lessard, M., Relton, J., Wong, G., Rivest, S., 2009. Powerful beneficial effects of macrophage colony-stimulating factor on beta-amyloid deposition and cognitive impairment in Alzheimer's disease. Brain 132, 1078–1092.

Brown, R.F., Valpiani, E.M., Tennant, C.C., Dunn, S.M., Sharrock, M., Hodgkinson, S., Pollard, J.D., 2009. Longitudinal assessment of anxiety, depression, and fatigue in people with multiple sclerosis. Psychol. Psychother. 82, 41–56.

Bulloch, K., Hamburger, R.N., Loy, R., 1982. Nest-building behavior in two cerebellar mutant mice: staggerer and weaver. Behav. Neural Biol. 36, 94–97.

Centonze, D., Muzio, L., Rossi, S., Cavasinni, F., De Chiara, V., Bergami, A., Musella, A., D'Amelio, M., Cavallucci, V., Martorana, A., et al., 2009. Inflammation triggers synaptic alteration and degeneration in experimental autoimmune encephalomyelitis. J. Neurosci. 29, 3442–3452.

Chesselet, M.F., Delfs, J.M., 1996. Basal ganglia and movement disorders: an update. Trends Neurosci. 19, 417–422.

Compston, A., Coles, A., 2008. Multiple sclerosis. Lancet 372, 1502–1517.

Cosentino, M., Zaffaroni, M., Marino, F., Bombelli, R., Ferrari, M., Rasini, E., Lecchini, S., Ghezzi, A., Frigo, G., 2002. Catecholamine production and tyrosine hydroxylase expression in peripheral blood mononuclear cells from multiple sclerosis patients: effect of cell stimulation and possible relevance for activation-induced apoptosis. J. Neuroimmunol. 133, 233–240.

Cryan, J.F., Mombereau, C., Vassout, A., 2005. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci. Biobehav. Rev. 29, 571–625.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat. Rev. Neurosci. 9, 46–56.

David, D.J., Renard, C.E., Jolliet, P., Hascoët, M., Bourin, M., 2003. Antidepressant-like effects in various mice strains in the forced swimming test. Psychopharmacology (Berl.) 166, 373–382.

Feinstein, A., 2007. Neuropsychiatric syndromes associated with multiple sclerosis. J. Neurol. 254, II73–II76.

Felger, J.C., Miller, A.H., 2012. Cytokine effects on the basal ganglia and dopamine function: the subcortical source of inflammatory malaise. Front. Neuroendocrinol. 33, 315–327.

Filali, M., Lalonde, R., Rivest, S., 2009. Cognitive and non-cognitive behaviors in an APPswe/PS1 bigenic model of Alzheimer's disease. Genes Brain Behav. 8, 143–148.

Garcia-Ovejero, D., González, S., Paniagua-Torija, B., Lima, A., Molina-Holgado, E., De Nicola, A.F., Labombarda, F., 2014. Progesterone reduces secondary damage, preserves white matter, and improves locomotor outcome after spinal cord contusion. J. Neurotrauma 31, 857–871.

Geurts, J.J., Barkhof, F., 2008. Grey matter pathology in multiple sclerosis. Lancet Neurol. 7, 841–851.

Grasselli, G., Rossi, S., Musella, A., Gentile, A., Loizzo, S., Muzio, L., Di Sanza, C., Errico, F., Musumeci, G., Haji, N., et al., 2013. Abnormal NMDA receptor function exacerbates experimental autoimmune encephalomyelitis. Br. J. Pharmacol. 168, 502–517.

Haji, N., Mandolesi, G., Gentile, A., Sacchetti, L., Fresegna, D., Rossi, S., Musella, A., Sepman, H., Motta, C., Studer, V., et al., 2012. TNF-α-mediated anxiety in a mouse model of multiple sclerosis. Exp. Neurol. 237, 296–303.

Hamers, F.P., Koopmans, G.C., Joosten, E.A., 2006. CatWalk-assisted gait analysis in the assessment of spinal cord injury. J. Neurotrauma 23, 537–548.

Imitola, J., Chitnis, T., Khoury, S.J., 2005. Cytokines in multiple sclerosis: from bench to bedside. Pharmacol. Ther. 106, 163–177.

Kawagoe, K.T., Wightman, R.M., 1994. Characterization of amperometry for in vivo measurement of dopamine dynamics in the rat brain. Talanta 41, 865–874.

Koo, J.W., Duman, R.S., 2008. IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proc. Natl. Acad. Sci. U. S. A. 105, 751–756.

Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. Nature 455, 894–902.

MacKenzie-Graham, A., Rinek, G.A., Avedisian, A., Gold, S.M., Frew, A.J., Aguilar, C., Lin, D.R., Umeda, E., Voskuhl, R.R., Alger, J.R., 2012. Cortical atrophy in experimental autoimmune encephalomyelitis: in vivo imaging. Neuroimage 60, 95–104.

Mandolesi, G., Musella, A., Gentile, A., Grasselli, G., Haji, N., Sepman, H., Fresegna, D., Bullitta, S., De Vito, F., Musumeci, G., et al., 2013. Interleukin-1β alters glutamate transmission at purkinje cell synapses in a mouse model of multiple sclerosis. J. Neurosci. 33, 12105–12121.

Markianos, M., Koutsis, G., Evangelopoulos, M.E., Mandellos, D., Karahalios, G., Sfagos, C., 2009. Relationship of CSF neurotransmitter metabolite levels to disease severity and disability in multiple sclerosis. J. Neurochem. 108, 158–164.

Marrie, R.A., Horwitz, R., Cutter, G., Tyry, T., Campagnolo, D., Vollmer, T., 2009. The burden of mental comorbidity in multiple sclerosis: frequent, underdiagnosed, and undertreated. Mult. Scler. 15, 385–392.

Meng, C., Brandl, F., Tahmasian, M., Shao, J., Manoliu, A., Scherr, M., Schwerthöffer, D., Bäuml, J., Förstl, H., Zimmer, C., et al., 2014. Aberrant topology of striatum's connectivity is associated with the number of episodes in depression. Brain 137, 598–609.

Mercuri, N.B., Bonci, A., Calabresi, P., Stefani, A., Bernardi, G., 1995. Properties of the hyperpolarization-activated cation current Ih in rat midbrain dopaminergic neurons. Eur. J. Neurosci. 7, 462–469.

Mori, F., Nisticò, R., Mandolesi, G., Piccinin, S., Mango, D., Kusayanagi, H., Berretta, N., Bergami, A., Gentile, A., Musella, A., et al., 2014. Interleukin-1β promotes long-term potentiation in patients with multiple sclerosis. Neuromolecular Med. 16, 38–51.

Murphy, K.T., Chee, A., Trieu, J., Naim, T., Lynch, G.S., 2012. Importance of functional and metabolic impairments in the characterization of the C-26 murine model of cancer cachexia. Dis. Models Mech. 5, 533–545.

Musgrave, T., Benson, C., Wong, G., Browne, I., Tenorio, G., Rauw, G., Baker, G.B., Kerr, B.J., 2011a. The MAO inhibitor phenelzine improves functional outcomes in mice with experimental autoimmune encephalomyelitis (EAE). Brain Behav. Immun. 25, 1677–1688.

Musgrave, T., Tenorio, G., Rauw, G., Baker, G.B., Kerr, B.J., 2011b. Tissue concentration changes of amino acids and biogenic amines in the central nervous system of mice with experimental autoimmune encephalomyelitis (EAE). Neurochem. Int. 59, 28–38.

Nakano, K., Higashi, T., Hashimoto, K., Takagi, R., Tanaka, Y., Matsushita, S., 2008. Antagonizing dopamine D1-like receptor inhibits Th17 cell differentiation: preventive and therapeutic effects on experimental autoimmune encephalomyelitis. Biochem. Biophys. Res. Commun. 373, 286–291.

Nisticò, R., Mango, D., Mandolesi, G., Piccinin, S., Berretta, N., Pignatelli, M., Feligioni, M., Musella, A., Gentile, A., Mori, F., et al., 2013. Inflammation subverts hippocampal synaptic plasticity in experimental multiple sclerosis. PLoS One 8, e54666.

Norden, D.M., Bicer, S., Clark, Y., Jing, R., Henry, C.J., Wold, L.E., Reiser, P.J., Godbout, J.P., McCarthy, D.O., 2014. Tumor growth increases neuroinflammation, fatigue and depressive-like behavior prior to alterations in muscle function. Brain Behav. Immun. http://dx.doi.org/10.1016/j.bbi.2014.07.013.

Peruga, I., Hartwig, S., Thone, J., Hovemann, B., Gold, R., Juckel, G., Linker, R.A., 2011. Inflammation modulates anxiety in an animal model of multiple sclerosis. Behav. Brain Res. 220, 20–29.

Petit-Demouliere, B., Chenu, F., Bourin, M., 2005. Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl.) 177, 245–255.

Pollak, Y., Orion, E., Goshen, I., Ovadia, H., Yirmiya, R., 2002. Experimental autoimmune encephalomyelitis-associated behavioral syndrome as a model of 'depression due to multiple sclerosis'. Brain Behav. Immun. 16, 533–543.

Pollak, Y., Ovadia, H., Goshen, I., Gurevich, R., Monsa, K., Avitsur, R., Yirmiya, R., 2000. Behavioral aspects of experimental autoimmune encephalomyelitis. J. Neuroimmunol. 104, 31–36.

Pollak, Y., Ovadia, H., Orion, E., Yirmiya, R., 2003. The EAE-associated behavioral syndrome: II. Modulation by anti-inflammatory treatments. J. Neuroimmunol. 137, 100–108.

Ripoll, N., David, D.J., Dailly, E., Hascoët, M., Bourin, M., 2003. Antidepressant-like effects in various mice strains in the tail suspension test. Behav. Brain Res. 143, 193–200.

Rossi, S., Muzio, L., De Chiara, V., Grasselli, G., Musella, A., Musumeci, G., Mandolesi, G., De Ceglia, R., Maida, S., Biffi, E., Pedrocchi, A., Menegon, A., Bernardi, G., Furlan, R., Martino, G., Centonze, D., 2010. Impaired striatal GABA transmission in experimental autoimmune encephalomyelitis. Brain Behav. Immun. 25, 947–956.

Rossi, S., Furlan, R., De Chiara, V., Motta, C., Studer, V., Mori, F., Musella, A., Bergami, A., Muzio, L., Bernardi, G., et al., 2012. Interleukin-1β causes synaptic hyperexcitability in multiple sclerosis. Ann. Neurol. 71, 76–83.

Sager, T.N., Kirchhoff, J., Mørk, A., Van Beek, J., Thirstrup, K., Didriksen, M., Lauridsen, J.B., 2010. Nest building performance following MPTP toxicity in mice. Behav. Brain Res. 208, 444–449.

Scarponi, M., Bernardi, G., Mercuri, N.B., 1999. Electrophysiological evidence for a reciprocal interaction between amphetamine and cocaine-related drugs on rat midbrain dopaminergic neurons. Eur. J. Neurosci. 11, 593–598.

Suh, Y., Motl, R.W., Mohr, D.C., 2010. Physical activity, disability, and mood in the early stage of multiple sclerosis. Disabil. Health J. 3, 93–98.

Surmeier, D.J., Ding, J., Day, M., Wang, Z., Shen, W., 2007. D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. Trends Neurosci. 30, 228–235.

Svenningsson, P., Nishi, A., Fisone, G., Girault, J.A., Nairn, A.C., Greengard, P., 2004. DARPP-32: an integrator of neurotransmission. Annu. Rev. Pharmacol. Toxicol. 44, 269–296.

Takao, K., Kobayashi, K., Hagihara, H., Ohira, K., Shoji, H., Hattori, S., Koshimizu, H., Umemori, J., Toyama, K., Nakamura, H.K., et al., 2013. Deficiency of schnurri-2, an MHC enhancer binding protein, induces mild chronic inflammation in the brain and confers molecular, neuronal, and behavioral phenotypes related to schizophrenia. Neuropsychopharmacology 38, 1409–1425.

Tao, G., Datta, S., He, R., Nelson, F., Wolinsky, J.S., Narayana, P.A., 2009. Deep gray matter atrophy in multiple sclerosis: a tensor based morphometry. J. Neurol. Sci. 282 (1–2), 39–46.

Zhu, C.B., Blakely, R.D., Hewlett, W.A., 2006. The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters. Neuropsychopharmacology 31, 2121–2131.