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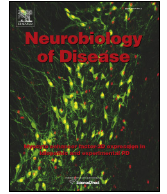

Interferon-γ causes mood abnormalities by altering cannabinoid CB1 receptor function in the mouse striatum

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A B S T R A C T

Interferon-γ (IFN-γ) has been implicated in the pathogenesis of multiple sclerosis (MS) and in its animal model, experimental autoimmune encephalomyelitis (EAE). The type-1 cannabinoid receptors (CB1Rs) are heavily involved in MS pathophysiology, and a growing body of evidence suggests that mood disturbances reflect specific effects of proinflammatory cytokines on neuronal activity. Here, we investigated whether IFN-γ could exert a role in the anxiety- and depressive-like behavior observed in mice with EAE, and in the modulation of CB1Rs. Anxiety and depression in fact are often diagnosed in MS, and have already been shown to depend on cannabinoid system. We performed biochemical, behavioral and electrophysiological experiments to assess the role of IFN-γ on mood control and on synaptic transmission in mice. Intracerebroventricular delivery of IFN-γ caused a depressive- and anxiety-like behavior in mice, associated with the selective dysfunction of CB1Rs controlling GABA transmission in the striatum. EAE induction was associated with increased striatal expression of IFN-γ, and with CB1R transmission deficits, which were rescued by pharmacological blockade of IFN-γ. IFN-γ was unable to replicate the effects of EAE on excitatory and inhibitory transmission in the striatum, but mimicked the effects of EAE on CB1R function in this brain area. Overall these results indicate that IFN-γ exerts a relevant control on mood, through the modulation of CB1R function. A better understanding of the biological pathways underling the psychological disorders during neuroinflammatory conditions is crucial for developing effective therapeutic strategies.

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1. Introduction

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease, and it is one of the most common causes of neurological disability in young adults. While the etiology of MS remains undetermined, an inflammatory cascade in the central nervous system (CNS) is thought to contribute to the pathological mechanisms of this disease (Compston and Coles, 2008; Ontaneda et al., 2016). The reduction in neuronal integrity and function that affects the grey matter compartment is thought to be the key process that leads to irreversible disability and cognitive impairment in MS (Calabrese and Gallo, 2009; De Stefano et al., 2003). In recent years, soluble inflammatory cytokines have been proposed as determinants of early alterations of the neuronal compartment occurring in this disorder (Mandolesi et al., 2015).

Among others, the pro-inflammatory cytokine interferon-gamma (IFN-γ) has been implicated in the pathogenesis of MS and of its animal model, experimental autoimmune encephalomyelitis (EAE), although the current literature did not convincingly determine the role of this cytokine in the disease (Arellano et al., 2015; Ottum et al., 2015). Significantly higher levels of IFN-γ has been reported in the CNS during inflammatory events in MS (Cannella and Raine, 1995; Kahl et al., 2002) and in EAE (Gardner et al., 2013; Hidaka et al., 2014), and clinical evidence showed that MS patients treated with recombinant IFN-γ in clinical trials developed more severe inflammation (Panitch et al., 1987). On the other hand, EAE studies using IFN-γ knockout (IFN-γ −/−) mice or neutralizing monoclonal antibodies directed against IFN-γ (anti–IFN-γmAb) showed that EAE was exacerbated under these conditions (Mangalam et al., 2014; Sosa et al., 2015). Together, these findings indicate that IFN-γ may either alleviate or worsen MS and EAE diseases.

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Some consensus exists on the involvement of IFN-γ in psychological comorbidities associated with MS. IFN-γ was reported as one of the cytokines involved in depressive behavior in humans and rodents (Himmerich et al., 2010; Lichtblau et al., 2013; O'Connor et al., 2009; Seidel et al., 1996), and a significant positive correlation has been demonstrated between IFN-γ and depressive symptoms in MS patients. Depression, which is a common symptom in MS patients (Feinstein et al., 2014; Turner et al., 2016), has been in fact associated with both increases in IFN-γ mRNA levels in whole blood, as well as in subclasses of T cells in relapsing-remitting MS patients (Joffe, 2005; Kahl et al., 2002; Mohr and Genain, 2004; Pokryszko-Dragan et al., 2012).

The neuronal mechanism by which IFN-γ affects mood during central inflammatory conditions is however unknown. To fill this gap, here, we performed neurophysiological recordings from striatal neurons since the dorsal striatum plays a central role also in cognitive and emotional functions (White and Salinas, 2003; Balleine et al., 2007). The type-1 cannabinoid receptors (CB1Rs) are heavily involved in MS pathophysiology, and a growing body of evidence suggests that mood disorders reflect specific effects of cytokines on neuronal activity. We have previously demonstrated that the loss of sensitivity of CB1Rs controlling inhibitory transmission represents a synaptic counterpart of the anxious-depressive state induced by chronic psychoemotional stress (Rossi et al., 2008), and by central inflammation in the striatum (Gentile et al., 2016; Rossi et al., 2012a), a brain area that contains high levels of CB1Rs (Szabo et al., 1998; Gerdeman and Lovinger, 2001; Huang et al., 2001; Piomelli, 2003; Andersson et al., 2005; Ade and Lovinger, 2007; Centonze et al., 2007a, 2007b).

Here, we report that intracerebroventricular (icv) delivery of IFN-γ causes anxious-depressive behaviors in mice, by altering the sensitivity of cannabinoid CB1Rs in specific subsets of striatal synapses. We also found that IFN-γ effect on CB1R function is relevant for the pathophysiology of mood disorders associated with EAE, supporting the concept that inflammatory cytokines are involved in emotional disturbances in this neuroinflammatory condition and, possibly, in MS by interacting with the endocannabinoid system.

2. Materials and methods

2.1. Experimental animals

Six- to eight-week-old female 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. All the efforts were made to minimize the number of animals utilized and their suffering. Animal experiments were carried out according to the Internal Institutional Review Committee, the European Directive 2010/63/EU and the European Recommendations 526/2007 and the Italian D.Lgs 26/2014.

2.2. In vivo treatments

Mice ($n = 8$–12) were anesthetized by intraperitoneal injection of tiletamine/zolazepam (80 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus with mouse adapter and lateral ear bars for surgery. The mice were implanted into the left lateral ventricle with 7-mm-long bilateral stainless steel cannulae using the following coordinates relative to bregma: $AP = -0.4$ mm; $L = +1.0$ mm; DV from the cortical surface $= -1.2$ mm, according to the mouse brain atlas (Franklin and Paxinos, 1997). After implantation of the intracerebral cannula, mice were housed in isolated cages endowed with special bedding (TEK-FRESCH, Harlan). After one week from cannulae implantation, IFN-γ (0,2 μg/mouse dissolved in PBS; R&D Systems) or vehicle were administered in vivo by a single intracerebroventricular (icv) injection. To this aim, an 8-mm injection needle connected with polyethylene tubing to a 2.0-μl Hamilton syringe was inserted into the guide cannula. A volume of 2 μl of IFN-γ or saline solution was infused. Each infusion lasted for 1 min, followed by 3 additional minutes with the needle left in place to allow diffusion. Behavioral or electrophysiological experiments were performed 24 h after the icv injection.

2.3. EAE induction

After 2 weeks of acclimatization, EAE was induced in 7–8-week female animals. Mice were injected subcutaneously at the flanks with 200 μg of myelin oligodendrocyte glycoprotein p35–55 (MOG35–55) emulsion to induce EAE by active immunization (Centonze et al., 2009; Musella et al., 2014). The emulsion was prepared under sterile conditions using MOG35–55 (>85% purity, Espikem, Florence, Italy) in complete Freund's adjuvant (CFA, Difco), and Mycobacterium tuberculosis H37Ra (8 mg/ml; strain H37Ra, Difco, Lawrence, KS, USA) emulsified with phosphate buffered saline (PBS). The control emulsion was prepared the same way without MOG35–55 for the control group (CFA group). All animals were injected with 500 ng pertussis toxin (Sigma, St. Louis, MO, USA) intravenously on the day of immunization and 2 days later according to standard protocols of EAE induction. In order to verify the appearance of clinical symptoms, animals were weighed and scored daily according to the following scale: 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness; 3, hind limb paresis; 4, complete bilateral hind limb paralysis; 5, death due to EAE. Intermediate clinical signs were scored by adding 0.5 value (Centonze et al., 2009). All the efforts were made to minimize their suffering; in particular, when animals experienced complete hind limb paralysis, moistened food and water were made easily accessible to the animals on the cage floor. In some circumstances, paralyzed mice received glucose solution by subcutaneous injection or food by gavage until the day of the sacrifice. In the rare presence of a moribund animal, euthanasia was provided.

2.4. 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). Nest building ability was valuated 12 h after icv injections, while LDT and TST were performed 24 h after icv treatment. 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.

2.4.1. Nest building test (NBT)

To evaluate the ability of nest construction, mice were individually housed 1 h before the onset of dark phase in a clean cage overnight aside a pre-weighted roll of cotton in the cage-top food hopper. The morning after the quantity of cotton used was measured. Furthermore, 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 cupshaped nest with sides, or (4) bowl- or cup-shaped nest with sides and a cover (Bulloch et al., 1982).

2.4.2. Light-dark test (LDT)

The light/dark test (LDT) is based on the innate aversion of rodents to brightly lit areas (Crawley, 1981). The test apparatus consisted of an open white compartment (30 × 20 × 20 cm, 300 lx) joined by a 3 × 3 cm opening to a dark compartment (15 × 20 × 20 cm, 0 lx) which was painted black and covered with a lid. The anxiogenic nature of the white compartment was increased by additional illumination from a 60-W angle poise lamp placed 45 cm above the center of the apparatus. Mice were placed into the dark compartment and latency to first exit, number of exits, number of rears and total time in the light compartment were recorded for 10 min.

The time spent in each chamber and the data showed in this work are referring to the last 5 min of the test and was recorded by ViewPoint video tracking software.

The score for the transition was assigned from the analysis of the video recordings, when the animal came out of the dark chamber with all 4 paws. The apparatus was cleaned with 10% ethanol after each trial to effectively remove the scent of the previously tested animal.

2.4.3. Tail suspension test (TST)

The tail suspension test has become one of the most widely used models for assessing potential antidepressant activity in rodents following treatment (Cryan et al., 2005). 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. The trial was conducted for 6 min during which a blinded observer scored the latency for the first immobility episode and total duration of immobility by using a stopwatch. The first episode of immobility was measured during the first 2 min, while the duration of immobility was estimated during the last 4 min of the test, using customize freeware software (ODLog). The mouse was considered immobile only when it hung passively and completely motionless. Mice that climbed their tails were eliminated from the analyses.

2.5. Electrophysiology

Mice were killed by cervical dislocation, and corticostriatal coronal slices (200 μm) were prepared from fresh tissue blocks of the brain with the use of a vibratome. A single slice was then transferred to a recording chamber and submerged in a continuously flowing artificial CSF (ACSF) (34 °C, 2–3 ml/min) gassed with 95% O_2–5% CO_2 as previously described (Gentile et al., 2016; Musella et al., 2014). The composition of the control ACSF was (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 Glucose, and 25 $NaHCO_3$. Recording pipettes were advanced towards individual striatal cells in the slice under positive pressure and visual control (WinVision 2000, Delta Sistemi, Italy) and, on contact, tight GΩ seals were made by applying negative pressure. The membrane patch was then ruptured by suction and membrane current and potential monitored using an Axopatch 1D patch clamp amplifier (Molecular Devices, Foster City, CA, USA). Whole-cell access resistances measured in voltage clamp were in the range of 5–20 MΩ. Whole-cell patch clamp recordings were made with borosilicate glass pipettes (1.8 mm o.d.; 2–3 MΩ), in voltage-clamp mode, at the holding potential (HP) of −80 mV. To study GABA-mediated spontaneous inhibitory postsynaptic currents (sIPSCs), the recording pipettes were filled with internal solution of the following composition (mM): 110 CsCl, 30 K+-gluconate, 1.1 EGTA, 10 HEPES, 0.1 CaCl2, 4 Mg-ATP, 0.3 Na-GTP. MK-801, and CNQX were added to the external solution to block, respectively, NMDA and non-NMDA glutamate receptors. To study glutamate-mediated spontaneous excitatory postsynaptic currents (sEPSCs), the recording pipettes were filled with internal solution of the following composition (mM): K+-gluconate (125), NaCl (10), $CaCl_2$, (1.0), $MgCl_2$ (2.0), 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA; 0.5), *N*-(2-hydroxyethyl)-piperazine-*N*-s-ethanesulfonic acid (HEPES; 19), guanosine triphosphate (GTP; 0.3), and Mg-adenosine triphosphate (Mg-ATP; 1.0), adjusted to pH 7.3 with KOH. Bicuculline (10 μM) was added to the perfusing solution to block $GABA_A$-mediated transmission. Only data from putative medium spiny projection neurons (MSNs) were included in the present study. These neuronal subtypes represent over 95% of the entire population of striatal neurons, and were identified for their morphological and electrophysiological properties. Fast-spiking GABAergic interneurons (putative parvalbumin (PV)-positive cells) and large aspiny interneurons (putative cholinergic interneurons) were recognized for their typical firing activity and somatic size (Kawaguchi et al., 1997; Koos and Tepper, 1999), and discarded. Synaptic events were stored by using P-CLAMP 9 (Axon Instruments) and analyzed offline on a personal computer with Mini Analysis 5.1 (Synaptosoft, Leonia, NJ, USA) software. The detection threshold of sEPSCs and sIPSCs was set at twice the baseline noise. The fact that no false events would be identified was confirmed by visual inspection for each experiment. Offline analysis was performed on spontaneous synaptic events recorded during fixed time epochs (1–2 min), sampled every 2–3 min (5–12 samplings). Only cells that exhibited stable frequencies in control (<20% changes during the control samplings) were taken into account. For each type of experiment at least four distinct animals were employed from each experimental group. Throughout the text "n" in electrophysiological experiments refers to the number of cells. Drugs used for the electrophysiological experiments were first dissolved in water or DMSO (HU210 and 5′-iodoresiniferatoxin) and then in the bathing artificial cerebrospinal fluid to the desired final concentration. The concentrations of the various drugs were (in μM): bicuculline (10), CNQX disodium salt (10), MK-801 (30), HU-210 (1), 5′-iodoresiniferatoxin (I-RTX, 1), mevastatin (10) (Tocris Cookson, Bristol, UK); recombinant mouse IFN-γ (100 ng/ml; R&D Systems) and rabbit anti-murine IFN-γ (250 ng/ml; Peprotech, USA). In the experiments with drugs dissolved in DMSO, the control samplings were obtained during DMSO and artificial cerebrospinal fluid applications.

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

Twenty-one dpi EAE and CFA mice (at least 6 for each group) were sacrificed through cervical dislocation; then, both left and right striata were quickly removed and frozen until use. 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, 500 ng of total RNA was reverse-transcribed using miScript II RT Kit (Qiagen) according to the manufacturer's instructions; 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 ΔΔCt method. β-Actin was used as internal controls. The following primer sequences were used.

Ifng (NM_008337): 5′-TGAGTATTGCCAAGTTTGAGGTCA-3′ (sense); 5′-CGACTCCTTTTCCGCTTCCT-3′ (antisense); Actb (NM_007393): 5′-CCTAGCACCATGAAGATCAAGATCA-3′ (sense); 5′-AAGCCATGCCAATGTTGTCTCT-3′ (antisense).

2.7. Statistical analysis

Data were presented as mean ± SEM. Throughout the text "n" refers to the number of the cells, with the exception of behavioral experiments, where "n" refers to the number of animals. The significance level was established at $p < 0.05$. Two-sample comparisons were carried out using paired or unpaired the Student's *t*-test for parametric measures or Mann-Whitney test for nonparametric variables. Multiple comparisons were analyzed by one-way ANOVA for independent measures followed by Tukey's post hoc analysis.

3. Results

3.1. Central delivery of IFN-γ induces anxiety- and depression-like behavior in mice

The emotional status of mice was tested at the NBT (12 h post injection) and at the LDT and TST (24 h post injection) after a single icv injection of IFN-γ or of vehicle.

Firstly, we performed the NBT, a motivation-based test which assesses nesting ability that is a natural and instinctive behavior in rodents (Paumier et al., 2013). We observed a reduction of both cotton used (icv-IFN-γ-mice n = 12, 1.25 ± 0.33 g; icv-vehicle-mice n = 13, 2.05 ± 0.16 g; unpaired Student's *t*-test $p < 0.05$; Fig. 1A) and nesting scores in IFN-γ treated mice compared to vehicle group (icv-IFN-γ-

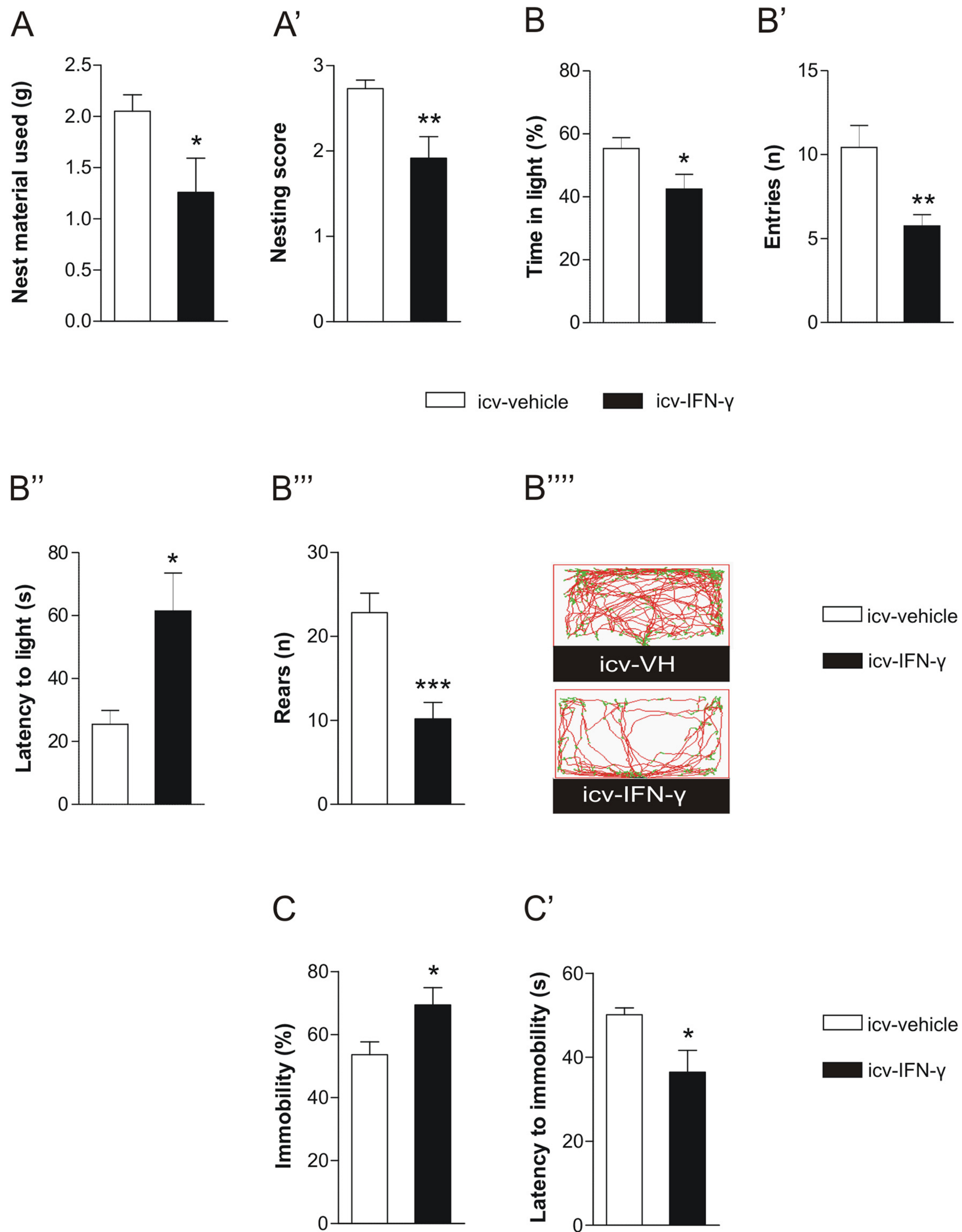

Fig. 1. Central delivery of IFN-γ induces anxiety- and depression-like behavior in mice. A–A′ Mice receiving icv injection of IFN-γ or vehicle were evaluated for their ability to construct nests (rated on a 4-point scale) to investigate motivation-based behavior. Bar graphs show a reduction in both cotton used (A) and in the quality of nest (A′) in mice treated with IFN-γ. B–B″′′ Exploratory behavior of mice was investigated by LDT. The graph shows that intracerebroventricular IFN-γ reduced the time spent in the light (B) and the number of entries in the light zone of the task (B′). The latency to enter in the light chamber (B″) and the vertical explorative behavior (B‴) were severely reduced in mice treated with IFN-γ compared to vehicle group at the LDT. B″′′ Representative video-recording tracking of mice performance in the light zone of the LDT apparatus. C–C′, Depressive-like behavior was investigated by TST. Icv injection of IFN-γ reduced the immobility (C) and the latency to the first bout of immobility (C′) at TST in comparison to vehicle group. Values are means ± SEM; statistical differences were analyzed by unpaired *t*-test or Mann-Whitney test for nesting score; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mice 1.92 ± 0.25; icv-vehicle-mice 2.73 ± 0.99; Mann Whitney non-parametric test $p < 0.05$, Fig. 1A′), indicating a reduced motivation-based activity.

At the LDT, the time spent in the light zone can be used as an index of anxiety-related responses, as anxiety reduces the time spent in the light (Bourin and Hascoet, 2003). We observed that mice receiving intracerebroventricularly IFN-γ spent a shorter time in the light compartment compared with vehicle-treated mice (icv-IFN-γ-mice n = 12 42.41 ± 4.72 s; icv-vehicle-mice n = 12, 55.32 ± 3.46 s; unpaired Student's *t*-test $p < 0.05$; Fig. 1B). IFN-γ affected also spontaneous

explorative behavior, as shown by the reduction of the frequency of chamber transitions (icv-IFN-γ-mice 5.75 ± 0.67; icv-vehicle-mice 10.42 ± 1.31; unpaired Student's *t*-test p < 0.01; Fig. 1B′), of the latency to enter in the light chamber (icv-IFN-γ-mice 61.42 ± 12.04 s; icv-vehicle-mice 25.42 ± 4.41 s; unpaired Student's *t*-test p < 0.05; Fig. 1B″), and of the number of vertical exploratory activity in IFN-γ treated-mice (icv-IFN-γ-mice 10.17 ± 1.95; icv-vehicle-mice 22.83 ± 2.29; unpaired Student's *t*-test p < 0.001; Fig. 1B‴).

Finally, at the TST mice receiving intracerebroventricularly IFN-γ were significantly different in terms of immobility compared to vehicle group (icv-IFN-γ-mice n = 9, 69.38 ± 5.5 s; icv-vehicle-mice n = 9, 53.64 ± 4.07 s; unpaired Student's *t*-test p < 0.05; Fig. 1C) and the latency to the first bout of immobility was increased in IFN-γ-treated mice (icv-IFN-γ-mice 36.44 ± 5.16 s; icv-vehicle-mice 50.11 ± 1.65 s; unpaired Student's *t*-test p < 0.05; Fig. 1C′), highlighting a depressive-like behavior induced by the cytokine.

3.2. Central delivery of IFN-γ causes CB1 receptor dysfunction in the striatum

In previous works, we demonstrated that the loss of sensitivity of CB1Rs controlling GABA synapses in the striatum represents a synaptic counterpart of the anxious-depressive state induced by chronic psychoemotional stress (Rossi et al., 2008), and by central inflammation (Gentile et al., 2016; Rossi et al., 2012a). In particular, we observed that even before the development of overt motor deficits, induction of EAE was associated with anxiety- and depression-like behaviors and such an effect seemed to be mediated, at least in part, by the downregulation of CB1Rs on striatal GABA synapses (Gentile et al., 2016).

Thus, we wondered whether also mood disturbances caused by IFN-γ involved the altered sensitivity of striatal CB1 receptors. The synaptic response to the CB1R agonist HU210 was therefore measured in brain slices taken from mice treated with icv vehicle or IFN-γ (n = 4 for both groups) 24 h after the treatment. In our experimental condition, HU210 reduced GABA-mediated sIPSC frequency in mice receiving icv vehicle (n = 10, paired Student's *t*-test p < 0.01), according to previous experiments obtained from untreated mice (Rossi et al., 2008; De Chiara et al., 2010). On the contrary, icv administration of IFN-γ was able to fully block the sensitivity of CB1Rs regulating GABA release in the striatum, abolishing the HU210-expected reduction of sIPSCs (n = 12, paired Student's *t*-test p > 0.05 compared with pre-HU210 values; Fig. 2A and B).

Altogether such results unravel as in vivo treatment with IFN-γ induces an anxiety- and depression-like behavior, and is able to fully block the sensitivity of CB1 receptors located on GABA synapses, mimicking the effects observed in response to chronic psychoemotional stress (De Chiara et al., 2010; Rossi et al., 2010, 2008) and to EAE condition (Gentile et al., 2016).

3.3. IFN-γ blocks CB1Rs sensitivity without altering spontaneous excitatory or inhibitory currents

Soluble mediators of inflammation have recently gained attention owing to their ability to modulate synaptic transmission and affect synaptic sensitivity to neurotransmitters (Gentile et al., 2016, 2015; Lai et al., 2006; Musella et al., 2016; Rossi et al., 2014a; Stellwagen and Malenka, 2006).

Thus, to further address the hypothesis that IFN-γ is able to affect central synaptic functioning, we evaluated the effect of this cytokine on neurophysiological parameters of both excitatory and inhibitory transmission in mouse brain slices, including their sensitivity to CB1R stimulation.

In striatal slices, incubation of IFN-γ for either 10 min (n = 10) or 120 min (n = 13) failed to affect sIPSC frequency or amplitude (frequency: vehicle 1.27 ± 0.11 Hz, IFN-γ 10 min 1.25 ± 0.13 Hz, IFN-γ 120 min 1.45 ± 0.10 Hz, one-way ANOVA p > 0,05; amplitude: vehicle 31.61 ± 2.1 pA, IFN-γ 10 min 30.8 ± 1.4 pA, IFN-γ 120 min 31.88 ± 1.7 pA, one-way ANOVA p > 0,05; Fig. 3A and B). In contrast, bath application of IFN-γ for 120 min was able to completely block the inhibitory effects of the CB1R agonist HU210 (% of pre HU210: vehicle 79.4 ± 4.0, n = 8, paired Student's *t*-test p < 0,01; IFN-γ 100.58 ± 4.8, n = 9, paired Student's *t*-test p > 0,05; Fig. 3C and D). Of note, IFN-γ failed to affect CB1R response to HU210 after 10 min of incubation (data not shown).

We also extended our investigations to the effects of IFN-γ on glutamatergic transmission. Application of IFN-γ for either 10 min (n = 16) or 120 min (n = 19) failed to alter frequency (vehicle 2.68 ± 0.19 Hz, IFN-γ 10 min 2.93 ± 0.21 Hz, IFN-γ 120 min 2.86 ± 0.25 Hz, one-way ANOVA p > 0,05; Fig. 3E), amplitude (vehicle 11.7 ± 0.7 pA, IFN-γ 10 min 12.9 ± 1.2 pA, IFN-γ 120 min 12.51 ± 0.93 pA, one-way ANOVA p > 0,05; Fig. 3F), and sensitivity to CB1R stimulation of sEPSCs (% of pre HU210: vehicle 81.3 ± 6.1; IFN-γ 2 h 82.5 ± 6.9; n = at least 10 neurons for each parameter, p < 0.05 for each parameter compared with pre-drug values; Fig. 3G).

These data indicate that IFN-γ acts on striatal synaptic transmission blocking the sensitivity of CB1Rs specifically located on GABAergic neurons, without altering spontaneous inhibitory and excitatory currents.

3.4. Increased expression of IFN-γ in the striatum of EAE mice

To see if IFN-γ could mediate some of the behavioral and synaptic effects described in EAE (Centonze et al., 2007a; Gentile et al., 2016; Rossi et al., 2011), we quantified, by means of qPCR technique, IFN-γ

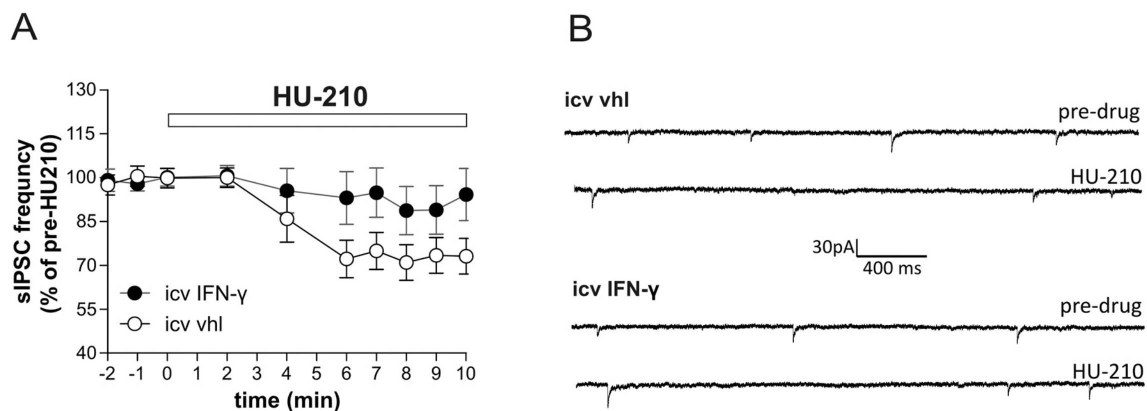

Fig. 2. Central administration of IFN-γ causes CB1 receptor dysfunction in the striatum. A–B, Intracerebroventricular treatment with IFN-γ was able to fully abolish the effect of HU210 on sIPSC frequency in control mice (p > 0.05), while HU210 caused the expected reduction of sIPSC frequency in mice receiving icv vehicle (p < 0.01). B, Examples of voltage-clamp recordings of sIPSCs before and during the application of HU210 in IFN-γ- and vehicle-treated mice. Values are means ± SEM; statistical differences were analyzed by paired *t*-test.

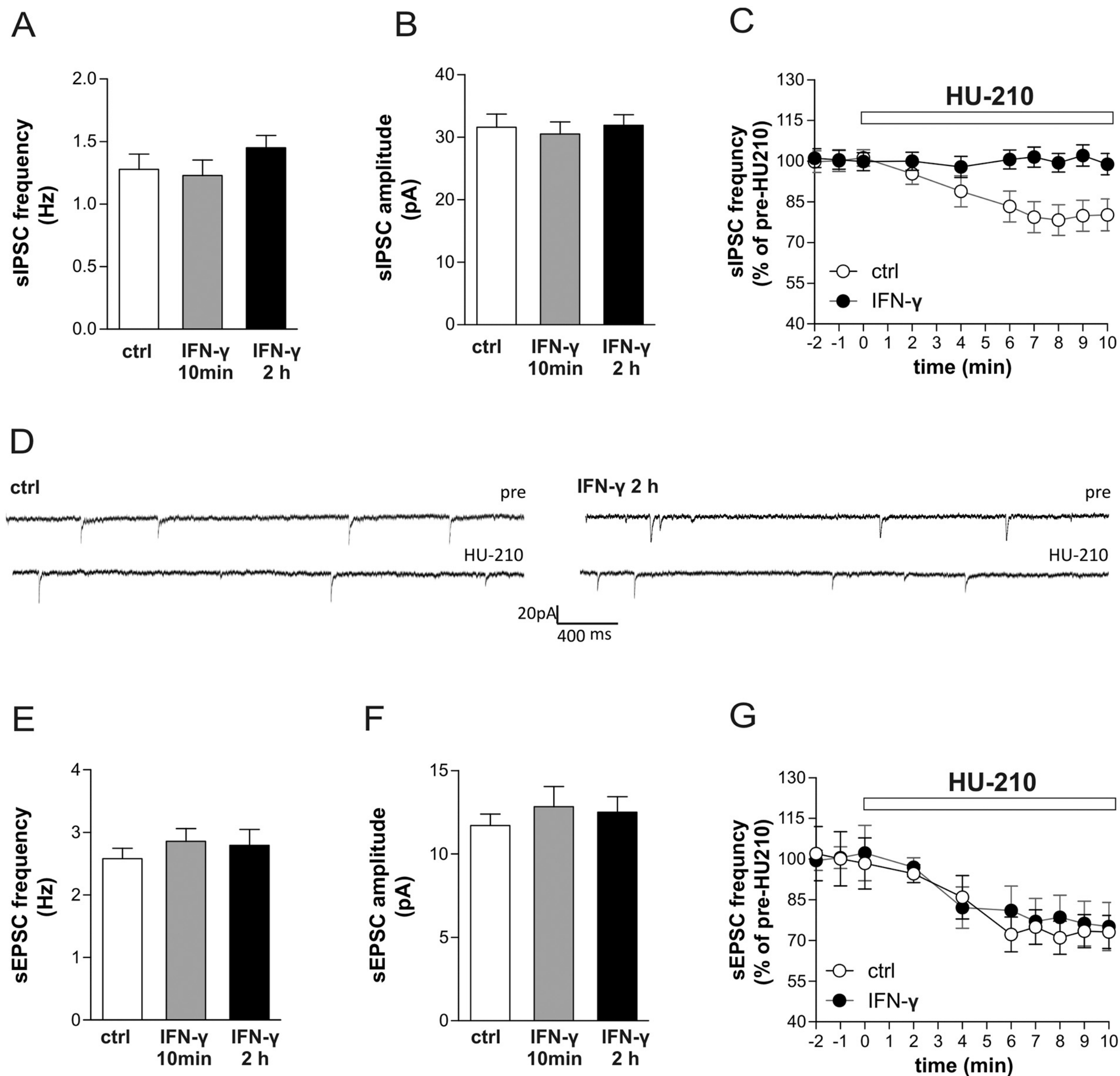

Fig. 3. Effects of IFN-γ on glutamate and GABA transmission recorded from striatal medium spiny neurons. A–B, Bath application of IFN-γ for both 10 min and 2 h failed to alter the frequency (A) and the amplitude (B) of spontaneous GABA-mediated currents (IPSCs). C, Stimulation of CB1Rs with HU210 reduced sIPSC frequency in control mice ($p < 0.01$). This effect was fully abolished in corticostriatal slices treated with IFN-γ for 120 min ($p > 0.05$). D, Examples of voltage-clamp recordings of sIPSCs before and during the application of HU210 in control slices and in slices treated with IFN-γ. E–G, Incubations of IFN-γ on corticostriatal slices did not affect the frequency and amplitude of the spontaneous excitatory transmission (sEPSCs, $p > 0.05$, E–F) and failed to alter HU210-mediated sEPSC frequency inhibition in MSN ($p < 0.05$ for both groups, G). Values are means ± SEM; statistical differences were analyzed by unpaired *t*-test or by paired *t*-test for HU-210 analysis.

mRNA in the striatum of EAE animals. As shown in Fig. 4, we detected more IFN-γ mRNA in EAE striatal extracts in comparison to the CFA group (CFA: 1.06 ± 0.18, n = 6; EAE 21 dpi: 16.37 ± 5.72, n = 7; $p < 0.05$ and; Fig. 4A).

These results support the notion that indeed IFN-γ could be involved in EAE-induced neuronal dysfunction in the striatum.

3.5. Blockade of IFN-γ signaling rescues CB1R function in EAE

To study if IFN-γ plays a role in EAE-induced synaptic abnormalities, we treated striatal slices of EAE mice with the anti-murine IFN-γ (anti–IFN-γmAb) before synaptic recordings. Application of anti–IFN-γ mAb in the bathing solution of EAE slice for 120 min was unable to reverse the deficits of sIPSC frequency (0.67 ± 0.05 Hz) or amplitude (33.13 ± 2.21 pA) typical of EAE (Rossi et al., 2011), but fully rescued CB1R functional defects. Indeed, sIPSC frequency was significantly reduced by HU210 in EAE slices treated with anti–IFN-γ mAb slices (n = 18) while it was unchanged in EAE untreated slices (n = 18; paired *t*-test: EAE-anti–IFN-γ mAb, $p < 0,01$; EAE-vhl, $p > 0,5$; Fig. 4B), as already reported (Gentile et al., 2016; Rossi et al., 2011).

3.6. IFN-γ effects on CB1Rs are different from those of IL-1β

We have previously reported that striatal CB1R activity is controlled through a mechanism dependent on transient receptor potential vanilloid 1 (TRPV1) (Maccarrone et al., 2008; Musella et al., 2010; Rossi et al., 2012a) and on lipid raft integrity (De Chiara et al., 2010; Rossi et al., 2012a), where CB1 receptors normally reside (Maccarrone et al., 2009; Oddi et al., 2012; Rimmerman et al., 2008). Indeed, IL-1β-mediated inhibition of striatal CB1R requires intact function of the TRPV1 channel and of lipid rafts (Rossi et al., 2012a).

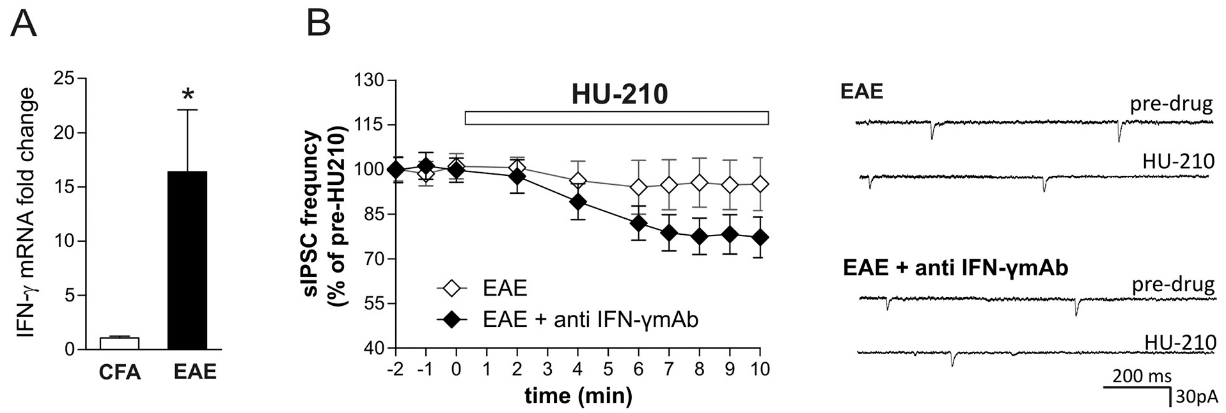

Fig. 4. IFN-γ is involved in EAE-induced CB1R dysfunction in the striatum. A, IFN-γ mRNA in EAE striatum is upregulated with respect to CFA control group ($p < 0.05$). B, HU210 was able to reduce striatal sIPSCs in EAE corticostriatal slices incubated with the anti-murine IFN-γ (anti–IFN-γmAb, $p < 0.01$), whereas it was ineffective in EAE untreated slices ($p > 0.05$). Electrophysiological traces on the right are examples of voltage-clamp recordings of sIPSCs before and during the application of HU210 in EAE slice incubated or not with IFN-γ. Values are means ± SEM; statistical differences were analyzed by unpaired *t*-test or by paired *t*-test for electrophysiology.

To see whether the mechanism by which IFN-γ regulates CB1R function is similar to that of the other major pro-inflammatory cytokine IL-1β, we measured the synaptic effect of IFN-γ in the presence of I-RTX, a receptor antagonist of TRPV1 channels. In our experimental condition, I-RTX was unable to prevent the loss of sensitivity of CB1R induced by IFN-γ, while TRPV1 blockade prevented the similar inhibitory effect that IL-1β exerted on these receptors (Rossi et al., 2012a) (Fig. 5A).

Furthermore, we also examined whether cholesterol synthesis plays a role in IFN-γ-induced CB1 dysfunction within the striatum. Bath application of mevastatin, a blocker of cholesterol synthesis already described to abrogate IL-1β-CB1R coupling in striatal slice (Rossi et al., 2012a), did not prevent the IFN-γ effect on CB1 activity (Fig. 5B).

4. Discussion

In the present investigation we demonstrated that administration of IFN-γ in the CNS induces anxiety- and depressive-like behavior in mice. Furthermore, we identified for the first time the relationship between IFN-γ and the dysfunction of CB1Rs controlling GABA transmission in the striatum, uncovering a possible synaptic underpinning of emotional control exerted by this cytokine. We also suggested that the increase of IFN-γ levels in the striatum of EAE mice and the resulting inhibition on CB1Rs contribute to the complex behavior of anxiety and depression, occurring in EAE animals and, possibly, MS.

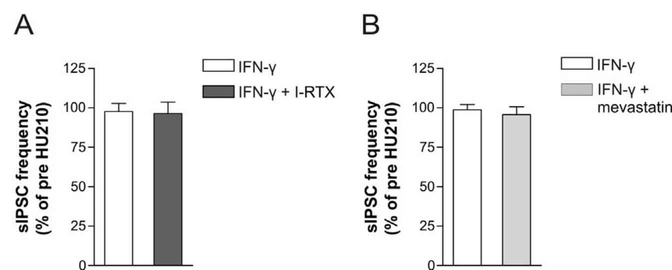

Fig. 5. IFN-γ effects on $CB1Rs_{(GABA)}$ are independent of TRPV1 and cholesterol synthesis. (A) The TRPV1 antagonist I-RTX, failed to prevent the loss of $CB1Rs_{(GABA)}$ sensitivity to HU210 in slices incubated with IFN-γ ($p > 0.05$ for both groups). B, The graph shows that mevastatin, a pharmacological blocker of cholesterol synthesis, did not prevent IFN-mediated inhibition of $CB1R_{(GABA)}$ ($p > 0.05$ for both groups). Values are means ± SEM; statistical differences were analyzed by paired *t*-test.

A growing amount of data point to the importance of the relationship between inflammation and depression. Patients suffering from major depressive disorder are known to have an increase in the production of specific cytokines (Dantzer et al., 2008; Dowlati et al., 2010; Lee and Kim, 2006; Raison et al., 2013; Slavich and Irwin, 2014). Immune signaling into the brain promotes an exacerbation of sickness and the development of symptoms of depression in vulnerable individuals, highlighting that pro-inflammatory cytokines that act on the brain might increase the risk of major depressive episodes (Dantzer et al., 2008). Among other cytokines, clinical evidence supports the involvement of IFN-γ in depressive behavior in humans and rodents.

O'Connor and colleagues demonstrated an essential role for interferon and TNF in depressive-like behaviors in mice, in response to chronic immune activation (O'Connor et al., 2009). Accordingly, side effects of interferon therapy include an increased depressive symptomatology (Loftis and Hauser, 2004; Piper et al., 2001; Riddell et al., 2001) and antidepressant therapy suppress the production of IFN-γ in human and rat auto-reactive T cells (Sommer et al., 1995).

Of note, mood disturbances in MS patients reflect specific effects of pro-inflammatory cytokines on neuronal function (Imitola et al., 2005; Kahl et al., 2002; Rossi et al., 2014b). Importantly, depressive symptoms positively correlated with IFN-γ mRNA levels in whole blood of MS patients during an acute attack (Kahl et al., 2002). Likewise, stimulated production of IFN-γ by peripheral T lymphocytes was related to depression and fatigue in MS patients (Mohr et al., 2001; Gold et al., 2011; Pokryszko-Dragan et al., 2012). Furthermore, in MS patients undergoing anti-depressive treatment, the decrease of depressive symptoms was associated with the reduction of IFN-γ production (Mohr et al., 2001; Mohr and Genain, 2004).

In line with the hypothesis that IFN-γ might contribute to the mood disorders associated with MS, we demonstrated that a single icv injection of IFN-γ induced an anxiety and depression-like behavior in mice, mimicking the behavioral defects typical of EAE mice (Gentile et al., 2016, 2015; Haji et al., 2012) and previously associated with the loss of striatal CB1R activity (Gentile et al., 2016). Accordingly, we found increased IFN-γ levels in the striatum of EAE mice, a brain area deeply involved in the control of complex cognitive and emotional functions (White and Salinas, 2003; Balleine et al., 2007) and, by means of electrophysiological recordings, we identified CB1R controlling GABA transmission as the main transmitter system modulated by IFN-γ. The interaction between the cannabinoid system and IFN-γ in EAE pathology was confirmed by blocking the IFN-γ signaling in EAE slices: in vitro application of the IFN-γ antibody in fact rescued the impairment of CB1R sensitivity in EAE slices. These data, in combination with the behavioral and electrophysiological results derived from icv delivery of

IFN-γ in healthy mice, further emphasize the concept that the dysfunction of striatal CB1Rs plays a crucial role in mood control during a variety of neuroinflammatory conditions.

To identify the molecular mechanism at the basis of CB1R functional alteration in the presence of IFN-γ, we investigated the role of TRPV1 channels and of lipid raft, which are known to play a fundamental role in CB1R activity (De Chiara et al., 2013; Rossi et al., 2012a) and that regulate the interplay between inflammatory cytokine and the endocannabinoid system (Rossi et al., 2014a). We previously demonstrated that the effect of IL-1β on striatal CB1 receptor sensitivity was indeed linked to cholesterol content in lipid rafts and to TRPV function (De Chiara et al., 2013; Rossi et al., 2012a). In the present study, however, either blockade of TRPV1 receptors or inhibition of cholesterol synthesis failed to affect the IFN-γ-mediated inhibition on CB1Rs, indicating the involvement of different players in IFN-γ-mediated action on cannabinoid control of neurotransmitter release. In line with this, several differences between the synaptic effect of IFN-γ and IL-1β should be noted. IFN-γ-mediated CB1R impairment was selective for cannabinoid on GABA neurons, whereas IL-1β induces a CB1 dysfunction on both GABA and glutamate terminals in the striatum (De Chiara et al., 2013; Rossi et al., 2012a). Moreover, IFN-γ was unable to alter spontaneous inhibitory and excitatory synaptic transmission, while IL-1β affects both glutamatergic and GABAergic synaptic properties (Nisticò et al., 2013; Rossi et al., 2012b, 2012c). Finally, in vitro, the incubation time necessary to induce the reported effects were significantly different for the two cytokines, as IFN-γ promoted its effect after a longer time interval compared to IL-1β, which was able to influence CB1R function already after a few minutes (De Chiara et al., 2013; Rossi et al., 2012a).

Different mechanisms may contribute to the described effect of IFN-γ on CB1R function, including changes of CB1R phosphorylation and of dopamine transmission. During EAE, in fact, CB1R phosphorylation is reduced in the striatum (Gentile et al., 2016), indicating an altered CB1R signaling and functioning. Furthermore, previous findings provide evidence that dopamine modulates striatal CB1R activity by a D2R-dependent mechanism (De Chiara et al., 2010; Gentile et al., 2015), and suggest that increased dopamine levels induce presynaptic rearrangements that recover CB1R function in EAE mice (Gentile et al., 2016). Further experiments are necessary to characterize whether the molecular mechanisms activated by IFN-γ are linked to CB1R phosphorylation or dopamine signaling.

Along with IFN-γ, other inflammatory molecules, such as TNF (Haji et al., 2012) and IL-1β (Gentile et al., 2016, 2015), have been demonstrated to contribute to the behavioral syndrome associated with EAE, leading to the idea that the emotional changes observed in EAE and, possibly, in MS, are the result of a complex interaction of multiple inflammatory mediators on neuronal circuits of brain areas involved in affective control.

5. Conclusions

The present work uncovers for the first time the synaptic underpinning of the emotional control exerted by IFN-γ, and provides further evidence that inflammatory cytokines modulate behavioral responses and the function of endocannabinoid system in EAE. Notably, despite its high clinical relevance, little is known about the pathophysiology of anxiety and depression in MS and other inflammatory diseases, and a better understanding of biological pathways underling this emotional disorders is crucial for developing effective therapeutic strategies.

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