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Electroconvulsive therapy is highly effective in neuropsychiatric disorders by unknown mechanisms. Microglial toxicity plays key role in neuroinflammatory and degenerative diseases, where there is critical shortage in therapies. This study examined the effects of electroconvulsive seizures (ECS) on chronic neuroinflammation and microglial neurotoxicity. Electric brain stimulation inducing full tonic-clonic seizures during chronic relapsing-progressive experimental autoimmune encephalomyelitis (EAE) reduced spinal immune cell infiltration, reduced myelin and axonal loss, and prevented clinical deterioration. Using the transfer EAE model we examined the effect of ECS on systemic immune response in donor mice versus ECS effect on CNS innate immune activity in recipient mice. ECS did not affect encephalitogenicity of systemic T cells, but targeted the CNS directly to inhibit T-cell induced neuroinflammation. In vivo and ex-vivo assays indicated that ECS suppressed microglial neurotoxicity, by reducing iNOS expression, nitric oxide and reactive oxygen species (ROS) production, and by reducing CNS oxidative stress. Microglia from ECS treated EAE mice expressed less T cell stimulatory and chemoattractant factors. Our finding indicate that Electroconvulsive therapy targets the CNS innate immune system to reduce neuroinflammation by attenuating microglial neurotoxicity. These findings signify a novel therapeutic approach for chronic neuroinflammatory, neuropsychiatric and neurodegenerative diseases.

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Electroconvulsive Stimulation Attenuates Chronic Neuroinflammation

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Abstract

Electroconvulsive therapy is highly effective in resistant depression by unknown mechanisms. Microglial toxicity was suggested to mediate depression, and plays key role in neuroinflammatory and degenerative diseases, where there is critical shortage in therapies. We examined the effects of electroconvulsive seizures (ECS) on chronic neuroinflammation and microglial neurotoxicity. Electric brain stimulation inducing full tonic-clonic seizures during chronic relapsing-progressive experimental autoimmune encephalomyelitis (EAE) reduced spinal immune cell infiltration, reduced myelin and axonal loss, and prevented clinical deterioration. Using the transfer EAE model we examined the effect of ECS on systemic immune response in donor mice versus ECS effect on CNS innate immune activity in recipient mice. ECS did not affect encephalitogenicity of systemic T cells, but targeted the CNS directly to inhibit T-cell induced neuroinflammation. In vivo and ex-vivo assays indicated that ECS suppressed microglial neurotoxicity, by reducing iNOS expression, nitric oxide and reactive oxygen species (ROS) production, and by reducing CNS oxidative stress. Microglia from ECS treated EAE mice expressed less T cell stimulatory and chemoattractant factors. Our finding indicate that Electroconvulsive therapy targets the CNS innate immune system to reduce neuroinflammation by attenuating microglial neurotoxicity. These findings signify a novel therapeutic approach for chronic neuroinflammatory, neuropsychiatric and neurodegenerative diseases.
**Introduction**

Neuroinflammation is considered a major mediator of brain injury in multiple chronic neurological and neuropsychiatric disorders, and presents an important therapeutic target for intervention (1). The brain's innate immune system, represented mainly by microglia, plays a leading role in chronic neuroinflammation-induced brain injury (2). Toxic microglial activation occurs both when the disease is inflicted by an autoimmune process, or secondary to pathologic cascade of events triggered by misfolded proteins in neurodegenerative diseases (3, 4). Activated microglia take on a pro-inflammatory phenotype, producing inflammatory cytokines, chemokines and free radicals, such as nitric oxide (NO) and reactive oxygen species (ROS) (5, 6). The confinement of the injurious neuroinflammatory disease process to the CNS, that is dominated by innate toxic microglia, occurring behind the blood brain barrier (BBB), creates a major challenge for developing targeted and effective therapies. This is exemplified in chronic-progressive multiple sclerosis (MS), in which the BBB is significantly less permeable (7), and immune pathogenesis is compartmentalized within the CNS (8-10). Microglia play a key role in the inflammatory damage in chronic MS (11). Indeed, current MS therapies targeting mainly the peripheral adaptive immune system are effective during the relapsing phase, but fail in progressive MS (12). The lack of therapies for chronic MS emphasizes the unmet need for developing treatments that target the CNS directly (13), modulating its toxic innate immune system.

Electro-convulsive therapy (ECT) is a CNS-targeted treatment that is highly effective in psychiatric disorders such as major depression and catatonic schizophrenia (14). Suggested mechanism(s) by which ECT affects the brain include increased hippocampal neurogenesis (15) and gliogenesis (16), elevation of trophic factors (BDNF, NGF, VEGF, FGF) (17-20), and modulation of various neurotransmitters (21). ECT may down-regulate immune activation (22), by inhibiting its markers of cellular activation (23), microgliosis (24, 25), altering TNFA (26) and IL6 (27) levels.
The therapeutic implications of these effects are unknown. As innate immune-pathogenic mechanisms may underlie also depression (28, 29), which is most responsive to ECT, we examined the effects of ECT on neuroinflammation.

Here we examined the therapeutic effects of ECT on chronic neuroinflammation in experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Repeated electroconvulsive seizures (ECS) arrested clinical deterioration in chronic EAE, decreased neuroinflammation, and reduced demyelination and axonal loss. Use of the transfer EAE model showed that ECS affects the CNS directly, not attenuating systemic adaptive immune encephalitogenicity. ECS’s therapeutic effect was mediated by reducing microglial toxicity, without transforming their phenotype. We conclude that electric brain stimulation attenuates chronic neuroinflammation –related CNS injury by reducing microglial toxicity.
Results

ECS markedly attenuates chronic neuroinflammation in chronic-progressive Biozzi EAE

In order to achieve effective electroconvulsive seizures, we established the electric threshold to cause a full tonic-clonic seizure, observed as static limb extension, followed by tonic-clonic limb and tail movements for 10-20 seconds. After seizures, mice appeared to be post-ictal (conscious but not moving willingly) for ~2 minutes. The electric threshold was 1.2 ± 0.18 mC (n=7) in Biozzi mice and 1.6 ± 0.31 mC (n=4) in SJL mice. In further experiments we treated mice with experimental autoimmune encephalomyelitis (EAE). We applied ECS with twice the average threshold, similar to common clinical practice in human patients and as published for other ECS protocols (30, 31).

Most EAE models are useful for studying systemic immune pathogenesis, but do not recapitulate pathologically and clinically the chronic aspects of MS (32). In the Biozzi mouse EAE model (33), mice undergo an acute relapse-remitting phase, followed by chronic relapsing and progressive disease. This chronic phase, developing usually after day 35 post-induction, is characterized by microgliosis, astrogliosis, chronic neuroinflammation, demyelinated lesions, remyelination, and progressive axonal loss that are suggestive of an immune-mediated neurodegenerative process (34). These characteristics make the Biozzi EAE model relevant for developing targeted therapies for chronic neuroinflammation (35). We therefore used it to examine the effect of ECS on neuroinflammation.

We first treated Biozzi mice with ECS (n=7) or sham treatment (n=6) at clinical presentation of the first relapse. Treatment was initiated on the first day of clinical signs in individual mice (tail drop, presenting 13-17 days post-induction (p.i.)), and four additional ECS sessions were applied on alternating days. The clinical effect was evaluated by calculating the cumulative score of the acute (first) relapse, spanning typically 15 days. Mild, but significant improvement was observed, as evident by reduced burden of disease during the first relapse (Figure 1A-B). The beneficial effect
was transient, as there was no difference in the severity of the progressive phase of EAE (Figure 1A).

The beneficial clinical effect of ECS on the acute relapse prompted us to examine the value of repeated ECS treatments on the course of chronic EAE. We therefore examined the effect of ECS treatment, delivered initially intensely in daily sessions, followed by long-term weekly ECS maintenance. To address the clinical situation of chronic disease, Biozzi EAE mice were subject to ECS sessions starting after the acute relapse, just prior to onset of the progressive phase. Three consecutive ECS sessions were started on days 35-37 p.i. (n=17) and were continued by weekly maintenance treatments. Two control groups were used: a sham treatment group (control, n=16) and a group receiving a sub-threshold electrical current of 0.4 mC (n=8), in order to account for any potential effects of an electric shock that does not stimulate distal nervous tissue, and in particular the spinal cord, where the neuroinflammatory process is most abundant. Significant and long-lasting clinical improvement was evident in mice treated by ECS (Figure 1C). These mice were less likely to relapse (Figure 1D), and their overall burden of disease was milder, as represented by a lower cumulative score during the chronic phase (days 35-95 p.i.) (Figure 1E). Mice treated with a subthreshold electric dose relapsed at the same rate as sham-treated mice, although this occurred several days later (probably due to the mere stress associated with the electric current). However, this group “caught up” with the sham-treatment group, and there was no difference in its mean cumulative score. Pathological analyses was performed after the long-term weekly maintenance ECS therapy (at the end of the experiment) on day 95 p.i., as shown on Figure 1C. Immunofluorescence staining showed that ECS-treated EAE mice exhibited significantly less T cell infiltration (59% reduction, P=0.03, Figure 2A-E), and less IBA1+ activated microglia/macrophages (44% reduction, P=0.02, Figure 2F-H) in the white matter of the spinal cord, as compared to control EAE mice. In addition, GFAP staining indicated a borderline significant (P=0.054) 35% reduction in astrogliosis in ECS-treated EAE mice as compared to control EAE mice (Figure 2I-K). As neuroinflammation
drives the demyelinating process and its associated axonal injury, we examined whether attenuation of neuroinflammation by ECS resulted in neuroprotection. Indeed, ECS treatment resulted in significantly less demyelination in the spinal cord, and nearly no axonal loss, as compared to sham-treated EAE mice (Figure 3A-N). We further quantified and compared the number of APC+ oligodendrocytes and NG2+ oligodendrocyte-progenitor cells. There was no difference in the number of oligodendrocytes between experimental groups (Figure 3O-Q). There was an increase in NG2+ cells in control EAE spinal cords (3.8-fold increase in relation to naïve spinal cord), possibly reflecting a proliferative response to the demyelinating process (Figure 3R, T). In ECS-treated EAE mice, there was just marginal increase in NG2+ cells (Figure 3S-T), in agreement with the marked attenuation of neuroinflammation and of demyelination. Thus, ECS attenuates chronic neuroinflammation in progressive EAE and prevents inflammation-induced demyelination and axonal loss in a clinical-relevant model chronic MS.

**ECS attenuates neuroinflammation by direct targeting of the CNS**

To investigate whether the therapeutic effect of ECS was mediated systemically via the peripheral immune system or directly on the CNS, we used the transfer EAE model in SJL mice. In this model, encephalitogenic auto-reactive T cells are isolated from lymph nodes of PLP-immunized mice (eg. donor mice), and injected to recipient naïve mice, which develop acute EAE. ECS therapy in donor mice may provide indication on its effect on peripheral immune responses, whereas ECS in recipient mice may provide indication on its direct effects on the CNS. Here, ECS sessions in PLP-immunized donor mice prior to lymph node cell collection did not have any effect on their encephalitogenicity, resulting in similar onset and severity of EAE in recipient mice (Figure 4A-B). Furthermore, Lymph node cells from ECS-treated and from sham-treated immunized mice exhibited similar in-vitro proliferative responses to the PLP-peptide autoantigen and to Concanavalin A (ConA, Figure 4C-D). This suggests that ECS effect is not mediated via the systemic adaptive autoimmune response, although we cannot rule out the possibility that incubation of lymph node
cells from ECS-treated donor mice with the autoantigen (or ConA) may have reversed a difference in the reactivity of T cells from the two experimental groups. When 3-consecutive daily ECS sessions were performed in recipient mice, starting at day 8 post-transfer (first day of clinical signs, when encephalitogenic T cells have entered the CNS), there was significant attenuation of EAE (Figure 4E-F). Thus, ECS probably does not induce its beneficial effect via the systemic immune system and attenuates neuroinflammation by direct targeting of the CNS.

We next examined the possibility that ECS-inhibitory effect on neuroinflammation was mediated by altering the blood-brain-barrier (BBB). BBB permeability (evaluated by using the Biocytin-TMR tracer) was compared between normal controls, untreated Biozzi EAE mice at the peak of acute (1st) relapse, and ECS- and sham- treated mice after the initial 3-consecutive days treatment sessions (eg. day 38 p.i., see Figure 1C). The tracer did not penetrate the CNS in naive mice (Figure 5A, E, I), whereas massive penetration was observed in EAE mice at the peak of acute relapse (Figure 5B, F, I). At day 38 (during remission), there was minimal penetrance of tracer (less than 0.02% of penetrance during peak of acute relapse), which did not differ between ECS- and sham treated mice (Figure 5C, D, G, H, I). Thus, ECS effect is mediated directly on the CNS, without any significant change in BBB permeability.

ECS modulates microglial immune response and neurotoxicity

In progressive MS, activated neurotoxic microglia play a central role in the pathogenesis of disease. Since ECS inhibited chronic EAE, affecting mostly the spinal cord, we hypothesized that electric stimulation of the cortex by ECS may be transmitted along the neuro-axis to modulate neuro-inflammation in distal spinal tracts. Furthermore, since ECS targeted the CNS directly, without affecting peripheral T-cell encephalitogenicity, we sought to examine its effect on the spinal cord innate immune system. iNOS+ microglia are known to mediate neurotoxicity in chronic neuroinflammatory and neurodegenerative disorders, with nitric oxide (NO) and reactive oxygen
species (ROS) playing a central role in microglial neurotoxicity (2). In chronic EAE mice (day 95 p.i.), there were significantly less iNOS+/IBA1+ cells in spinal white matter tracts of ECS-treated mice as compared to sham-treated mice (Figure 6A-G). To further characterize the effect of ECS on the innate immune response, we isolated CD11b+ cells from spinal cords of ECS or sham treated Biozzi EAE mice one day after the 3-day ECS treatment initiation (day 38 p.i.). The expression of iNos, pro-inflammatory and anti-inflammatory cytokines and of chemokines was examined by RT-PCR. ECS induced a 90% inhibition (P<0.001) of the highly expressed iNos mRNA in EAE mice (Figure 6H), and reduced the expression of Il2 (ΔRQ=-0.67, P=0.003) and Cxcl9 (ΔRQ=-0.41, P=0.001), that are involved in stimulating and attracting T cells (Figure 6I-J). These may explain the reduced T cell infiltration to the CNS in ECS-treated mice. In comparison to EAE mice, in ECS-treated EAE mice there was a trend for inhibition of Il1b (Figure 6K) and a non-significant effect on Cxcl10 (ΔRQ =0.07, P=0.3), Ccl2 (ΔRQ=-0.2, P=0.2 ), Ccl3 (ΔRQ=0.3, P=0.07 ), Ccl4 (ΔRQ=0.48, P=0.1), Il4 (ΔRQ=-0.35, P=0.3), Il10 (ΔRQ= -0.002, P=0.5) and Tgfβ (ΔRQ=-0.014, P=0.5). Of note, ECS treatment reduced iNos, Cxcl9 and Il1b expression, albeit not to the levels found in naïve animals (Figure 6 I-K). We then performed ex-vivo functional assays on freshly isolated CD11b+ cells from spinal cords of sham- and ECS-treated EAE mice on day 38 and examined by FACS analysis the level of IBA1 expression, as an activation marker. In ECS-treated EAE mice there was marked inhibition in the level of IBA1 expression (Figure 6L), with an increase in the population of IBA1low, on the expense of IBA1high. Furthermore, CD11b+ cells isolated from ECS-treated EAE mice produced significantly less NO and ROS (Figure 6M-N) than cells isolated from sham-treated EAE mice. FACS analysis indicated the existence of high and low ROS producing CD11b+ cell populations in EAE mice. ECS reduced the fraction of high ROS producing cells (Figure 6O). These results collectively suggest that ECS prevents a neurotoxic phenotype of microglia without affecting their basic immune functions, such as cytokine response. To further examine whether ECS prevents a neurotoxic phenotype of microglia, we evaluated the oxidative stress in EAE mice. Formation of
ROS causes degradation of lipids, resulting in Malondialdehyde (MDA) production, which can therefore serve as indicator of oxidative injury. To examine whether the reduction in iNOS+, ROS-producing microglia was associated with a reduction in oxidative stress, we stained for MDA in the spinal cords of EAE mice at the end of clinical follow-up (day 95 p.i.). In ECS-treated mice there was a significant decrease in MDA+ cells both in the white and gray matter of the spinal cords (Figure 7A-E). Thus, ECS reduces the neurotoxic phenotype of microglia/macrophages in EAE, as shown by the expression of neurotoxic markers, production of oxidants in functional assays, and in the pathologic outcome of tissue oxidative injury.
Discussion

We examined here the effects of brain stimulation by ECS on chronic active neuroinflammation using the EAE model in Biozzi mice, a model that is reminiscent of chronic MS. Our study provides the first demonstration that ECS inhibits neuroinflammation clinically and pathologically and protects from inflammation-driven neurodegeneration and reducing the extent of demyelination and axonal loss. Importantly, ECS was effective in a clinically relevant disease model, when administered after disease onset in an established disease, using a standard clinical protocol in terms of electrical dose and frequency of treatments. Furthermore, repeated ECS sessions produced an ongoing therapeutic effect that is crucial for chronic-active brain disorders. These observations make this mode of therapy and its mechanism of action a valid therapeutic target for clinical translation.

ECS attenuated major features of chronic neuroinflammation, as evident by reductions in T-cell infiltration, astrogliosis and microgliosis. The immunomodulatory effect of ECS was mediated in part by targeting of the CNS innate immune system and reducing microglial neurotoxicity. It is widely accepted that microglia affect neuronal function in health and disease (36), and that activated toxic microglia drive neurodegeneration in chronic neuroinflammatory and neurodegenerative diseases (11, 37). Our study provides a link between electrical activity and microglial function. We used here supra-threshold electric stimulation to transmit the therapeutic effect from the cortex to the spinal cord and showed that electric neurostimulation can drive microglial health and reverse their hyperactive neurotoxic phenotype to attenuate neuroinflammation. This is supported by recent studies that describe immune-modulatory effects in the spinal cord by transcutaneous electrical nerve stimulation (38) and in the brain by vagal nerve stimulation (39, 40). Indeed, several neuronal derived chemokines, cytokines, and neurotransmitters have been shown to regulate microglial functions (41, 42). Our findings are in agreement also with the wider concept of (neural) activity-dependent restoration of brain health, which is supported by observations of promotion of rewiring.
(43) and of oligodendrogenesis and myelination (44) by neuronal activity.

Current MS therapies target the peripheral adaptive immune system, modifying the relapsing-remitting phase of the disease. They are not useful in progressive MS (13), where systemic autoimmunity declines, and the disease is driven in a compartmentalized manner from within the CNS (9). In progressive MS, microglia seem to lose their normal homeostatic phenotype (45) and are activated throughout the CNS (46) producing toxic chemokines, cytokines, NO and ROS. The direct targeting of neurotoxic microglia by ECS underscores the pivotal role of activated microglia in driving CNS injury in chronic active EAE and MS (9, 47). ECS did not prevent the formation of highly encephalitogenic T cells, but rather affected brain innate immune cells and prevented microglial and innate immunity-mediated neuroinflammation, demyelination and axonal loss.

The implications of these findings address several neurodegenerative and neuropsychiatric diseases, in addition to MS. Clinically, ECT is the most effective treatment in depression, and is the treatment of choice in drug-resistant depression (48). There is increasing evidence that activated microglia play an important role in the pathogenesis of depression (27, 49). Thus, our findings provide an attractive mechanism by which ECT may affect and reverse depression. Also, microglial neurotoxicity has been shown to drive the neurodegenerative process in transgenic models of familial Alzheimer's disease (50). Importantly, dysfunctional microglia, such as that caused by TREM2 mutations also promote Alzheimer's disease pathogenesis (51, 52). These underscore the notion that both over-activation and suppression to a mal-functional state of microglia are deleterious to brain health, and that restoration of microglial homeostasis is the preferred therapeutic target, rather than their elimination or total inhibition. Our findings suggest that ECS may reduce microglial toxicity, without eliminating their ability to produce immune mediators. Importantly, ECS did not cause a general suppressive effect on microglia, nor a significant effect on cytokines associated with an M1 versus M2 phenotype. Rather, ECS reversed microglial neurotoxic phenotype, as indicated by reducing iNOS expression, NO and ROS production, and reducing the expression of Il2 and Cxcl9,
as T-cell stimulating and attracting cytokines. The lack of a general suppressive effect of ECS on microglial function, is important in enabling the still activated, ECS-treated microglia to participate in their homeostatic roles (53) as well as in regenerative processes (54). While we show a remarkable inhibitory effect of ECS on neuroinflammation, this does not rule out additional mechanisms of action, affecting the resilience of the various populations of brain cells.

In conclusion, electroconvulsive therapy induces an immunomodulatory therapeutic effect in a clinically relevant setting of experimental chronic multiple sclerosis. CNS microglia serve as a key therapeutic target for chronic-progressive MS, and modulation of their neurotoxicity by ECT may considerably reduce their neurotoxicity. These findings may bare implications to other neurodegenerative and neuropsychiatric diseases driven by microglial neurotoxicity, such as Alzheimer’s disease (50) and major depression (55).
Materials and Methods

Experimental Autoimmune Encephalomyelitis.

Biozzi ABH and SJL/J female mice (Envigo) were bred and grown under specific pathogen-free conditions. Animal experiments were approved by the IRB; MD-14-14089-4, MD-17-14988-5.

EAE was induced in 6-8 week old Biozzi ABH mice using spinal cord homogenate (SCH) as described (33, 56) and were scored daily as follows: 0- asymptomatic; 1- loss of tail tonicity; 2-difficulty in rolling over; 3- hind leg weakness or limping; 4- hind leg paralysis; 5- full four leg paralysis.

For transfer EAE, 6-7 week old female SJL/J mice were immunized with 175µg proteolipid protein (PLP) 139-151 peptide in 100µl saline and equal volume of CFA, containing 5mg/ml H37RA (Difco Laboratories). Ten days after immunization, lymph node cells (LNC) were harvested. 3x10^6 cells/ml were cultured for 72 hours in the presence of 100µg/ml PLP 139-151 peptide. 15x10^6 cells were injected intraperitoneally into recipient 6-7-week-old female SJL/J mice. EAE signs were observed clinically around day 8 p.i. Mice were followed clinically up to day 25 and scored as previously described (57).

Electro-convulsive seizures (ECS).

ECS was applied via ear clip electrodes using the Ugo Basile ECT unit (57800). ECS parameters were set on: Frequency – 100 Hertz, pulse width – 0.5 milliseconds, shock duration – 1 second. The electric threshold is the lowest dose causing a full seizure. Threshold was established by changing the current in intervals of 2 mA, starting at 8 mA. All ECS sessions were then given using a current of 24 mA equal to an electric dose of 2.4 mC (according to the equation: Q electric dose (milli-Coulombs mC) = I current (A) x PW pulse width (mS) x 2F frequency (Hz) x D duration (S) (58). All control mice were sham treated by attaching ear clip electrodes without applying a current.
Three experimental ECS protocols were used;

1. ECS was applied individually on the first day of clinical signs in EAE Biozzi mice, followed by four ECS treatments on alternating days.

2. ECS (and an additional control group receiving subthreshold electric dose of 0.4 mC (4 mA current) was applied to Biozzi mice starting on day 35 p.i. of EAE for three consecutive days (35-37 p.i.) followed by once weekly ECS treatment up to day 95.

3. In the transfer EAE model, SJL/J donor mice were subject to 3 consecutive ECS sessions on days 7-9, and LN were harvested on day 10 for culture and transfer experiments. In other experiments, recipient mice received three consecutive ECS treatments on days 8-10 post LNC injection (with onset of EAE clinical signs).

All experiments were repeated two to four times, number of repeats and the number of samples (n) in each experimental group are specified in figure legends.

**Histopathology**

Animals were anesthetized with pentobarbital and perfused with ice-cold PBS followed by cold 4% paraformaldehyde. Spinal cords were placed in OCT and deep frozen in dry ice. Serial 10μm longitudinal sections of spinal cords were prepared and immune-fluorescent stainings were performed as previously described (59). The following antibodies were used: anti-CD3 (Bio-Rad Laboratories, MCA1477), anti-IBA1 (Fujifilm Wako, 019-19741), anti-iNOS (Novus Biologicals, NBP2-22119), anti-NEUN (Millipore, MAB377), anti-APC (Ab-7, Calbiochem, OP80), anti-MDA (abcam, ab6463), anti-GFAP (Dako, Z0334), anti-NG2 (Millipore, AB5320), anti-MPB (Millipore, MAB386), anti-NF M (Chemicon International, AB1987). Goat anti-rabbit Alexa-fluor 488, Goat anti-mouse Alexa-fluor 488, Goat anti-rabbit Alexa-fluor 555, goat anti-rat Alexa-fluor 555 and goat anti-mouse Alexa-fluor 555 (1:200, Invitrogen) were used as secondary antibodies appropriately. Nuclear counterstain
was performed using DAPI (Vector Laboratories). Bielschowsky silver impregnation (for axons) and gold-black (for myelin) staining were performed as previously described (60).

**Quantification of axonal and myelin loss**

Analysis was performed on longitudinal sections ventral to the central canal, in a consistent manner. Degree of loss was assessed by measuring the area of injury out of total white matter (WM) area using ImageJ software analysis (ver. 1.51H, NIH, USA). Four sections, 60 microns apart were quantified per spinal cord, and average percent ± SEM of axonal or myelin area loss was calculated.

**Quantification of gliosis and inflammatory response.**

CD3+ T cells were counted along the meninges and white matter on both sides of the cervical spinal cord (cut uniformly in 7mm long longitudinal sections) and quantified as number of cells per section. IBA1+ cells, IBA1+iNOS+ cells, GFAP+ cells, APC+ cells and NG2+ cells were counted manually in WM and associated meninges in six microscopic fields (x40 power of objective x10 power of eyepiece) for each section. Four sections, 60 microns apart were quantified per mouse, followed by calculating group average cell number per microscopic field ± SEM. All experiments were conducted in a blinded manner, adhering to stereological principles.

**Blood brain barrier permeability**

Four Biozzi ABH mice groups (3 mice per group) were evaluated; Naïve mice, EAE mice at Peak of the first relapse (day 18 p.i.), and EAE mice at day 38 p.i. (remission) following three consecutive ECS or sham sessions. 1 mg of 5-(and 6)-tetramethylrhodamine biocytin (Biocytin-TMR, Invitrogen) diluted in 100ul PBS was injected per mouse into the tail vein. Penetration into the blood circulation was indicated by pink colorization of the ears within seconds. 30 minutes after injection animals were anesthetized and perfused as described above. Spinal cords were extracted, deep frozen in dry ice and serial 10μm longitudinal sections were prepared. Nuclear counterstain was performed.
using DAPI (Vector Laboratories).

Images (x40 power of objective x10 power of eyepiece) from white and gray matter were captured using identical laser intensity, exposure times and magnification in all cohorts. To set these parameters, livers from tracer injected (positive control) and non-injected mice (negative control) were used. Fluorescent intensity analysis was performed. Eight images per section, from four sections 60 microns apart were quantified (as Intensity Optical Density (IOD)) using image-pro plus software (Media Cybernetics), and the group average IOD per microscopic field ± SEM was calculated. All experiments were conducted in a blinded manner, adhering to stereological principles.

**Malondialdehyde (MDA) Quantification and analysis**

Designated fields in the SC were scanned and captured (x40 power of objective x10 power of eyepiece) using the Nikon-TL Imaging microscopic system, and all DAPI+ cells in each image were counted along the white matter and gray matter separately. 150-250 cells per each WM or GM area were counted in each image. The fraction of the MDA+ cells out of the total cell count was calculated. Four images (of sections 60 microns apart) were quantified per mouse, followed by calculating group average ± SEM. All experiments were conducted in a blinded manner.

**LNC proliferation assay**

LNCs from ECS or sham treated SJL/J mice were isolated 10 days after PLP immunization and cultured for 72-hour with Concanavalin A (2.5µg/ml, Sigma-Aldrich) or PLP 139-151 peptide (100µg/ml). Proliferation was assessed using BrdU incorporation as previously described (61).

**Microglia/macrophages isolation and FACS analysis**

Spinal cord tissue from Biozzi ABH EAE mice were dissociated to single cell suspension at day 38 p.i. following three consecutive days ECS or sham sessions, using the Neural Tissue Dissociation Kit (Miltenyi Biotec). Myelin was removed using Percoll (GE Healthcare) followed by
microglia/macrophages isolation using CD11b microbeads and MS columns (Miltenyi Biotec) according to manufacturer instructions. Degree of microglia/macrophages enrichment was assessed by consequent CD11b (BD Bioscience, M1/70) staining and FACS analysis (Beckman Coulter), in all experiments at least 75% of isolated cell expressed CD11b. IBA1 staining was performed with anti-IBA1 antibody (Fujifilm Wako, 019-19741) following staining with secondary antibody (goat anti-rabbit Alexa-fluor 555, 1:100, Invitrogen), using the fixation buffer and intracellular staining Perm Wash buffer (BioLegend) and analyzed by FACS (Beckman Coulter).

**NO and reactive oxygen species (ROS) measuring**

Spinal cord tissue from Biozzi ABH EAE mice were dissociated to single cell suspension at day 38 p.i. following three consecutive days ECS or sham sessions, and microglia/macrophages were isolated using CD11b microbeads as described above. Freshly isolated microglia/macrophages were seeded on poly-L-lysine–covered 96 well plates. Cells were activated over-night with LPS (2ng/ml, E. coli O111:B4, Sigma-Aldrich). NO production was assessed (on 250x10^5 cells/ well) using Greiss Reagent System according to manufacturer’s protocol (Promega, G2930) and quantified using ELISA plate reader (Tecan Spark 10M). ROS production was measured (on 50x10^5 cells/ well) using DCFDA dye according to manufacturer’s protocol (abcam, AB-ab113851) and quantified using ELISA plate reader (Beckman Coulter DTX 880 multimode detector). To measure intracellular ROS, freshly isolated microglia/macrophages were stained with DCFDA, and cell fluorescence was quantified using FACS.

**Real-Time PCR**

RNA was isolated from microglia/monocytes using RNeasy Plus Mini Kit (Qiagen). cDNA was generated from a concentration of 50 µg/ml RNA using qScript cDNA synthesis Kit (Quanta Biosciences). Reaction mixture included 1 µl of cDNA, 300 nM of appropriate forward and reverse primers (Agentek) and 5 µL PerfeCTA SYBR Green FastMix ROX (Quanta Biosciences) to a total
volume of 10 µL. Gene amplification was carried out using the StepOnePlus real-time PCR system (Applied Biosystems). Primers used:

*iNos* F: 5’ CCC-AGC-CTT-GCA-TCC-TCA-T; *iNos* R: 5’ ATG-CGG-CCT-CCT-TTG-AGC.


*Cxcl9* F: 5’ GCA-GTG-TGG-AGT-TCG-AGG-AA; *Cxcl9* R: 5’ CTG-TTT-GAG-GTC-TTT-GAG-GGA-TTT-G.


*Ccl2* F: 5’ ACG-TGT-TGG-CTC-AGC-CAG-AT; *Ccl2* R: 5’ CAG-CCT-ACT-CAT-TGG-GAT-CAT-CT.

*Ccl3* F: 5’ CCA-AGT-CTT-CTC-AGC-GCC-ATA-T; *Ccl3* R: 5’ TGG-AAT-CTT-CCG-GCT-GTA-GGA.

*Ccl4* F: 5’ CGT-GTC-TGC-CCT-CTC-TCT-CC; *Ccl4* R: 5’ GGA-GGG-TCA-GAG-CCC-ATT-G.


*Tgfb* F: 5’ CTG-AAC-CAA-GGA-GAC-GGA-ATA-CA; *Tgfb* R: 5’ GGG-CTG-ATC-CCG-TTG-ATT-T.
**Statistical analysis**

Statistical analysis was performed using SPSS, version 24. The statistical tests used in this manuscript were student’s one-way unpaired T test, three-way chi-square test or one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test, as appropriate. The test used was specified for each experiment in the figure legends. P value was considered significant < 0.05.

**Study approval**

Animal experiments were approved by the Hebrew University- Hadassah medical center IRB committee; MD-14-14089-4, MD-17-14988-5.
Author contributions

SG contributed by designing research studies, conducting experiments, acquiring data, analyzing data and writing the manuscript. NF contributed to designing research studies and conducting experiments. TBH contributed by designing research studies, analyzing data and writing the manuscript.

Financial Disclosures

Dr. Ben-Hur reports being a paid scientific consultant to Kadimastem, MAPI Pharma, Regenera Pharma, Sipnose, Stem Cell medicine and Medasense. There is no conflict of interest to this study. All other authors report no biomedical financial interests or potential conflicts of interest.

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Bibliography


Figures

Figure 1: ECS attenuates chronic EAE.

(A-B) ECS attenuates the first EAE relapse. Five ECS (n=7 mice) or sham (control, n=6 mice) treatments were performed on alternating days, starting on the first day of clinical deterioration, individually per each mouse. Average daily clinical scores show mild improvement in the clinical severity of the first relapse of EAE in ECS treated mice. Error bar – SD (A). The cumulative score of
the first relapse (lasting in average 15 days) was significantly lower in ECS-treated EAE mice (B). Experiment was repeated twice. P values calculated with Student’s unpaired T test.

(C-E) ECS attenuates chronic EAE. Biozzi EAE mice were treated with ECS (2.4 mC, n=16 mice), a sub-threshold current (0.4 mC, n=8 mice) or sham treatment (control, n=17 mice) starting prior to the second relapse. Treatments were given on three consecutive days (35-37 p.i.), followed by weekly maintenance treatments until day 95 p.i. Average daily clinical scores show that ECS (but not sub-threshold stimulation) improves the clinical course of chronic EAE (C). error bars – SEM. Experiment was repeated 4 times. ECS significantly reduced the relapse rate as compared to both sham and subthreshold treatment groups. Relapse was determined by an increase of >1 point in clinical score at any time-point between day 35-95 p.i. (D). P values calculated by 3-way chi-square test. ECS significantly reduced the cumulative clinical score of days 35-95 p.i. Dots represent the mean cumulative score of the four individual experiments (E). P value was calculated by using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $f(2,8) = 6.3$, $p=0.023$. Box-and-whisker plots show quartiles with median and minima and maxima at the bottom and top whiskers, respectively.
Figure 2: ECS inhibits chronic neuroinflammation.

Pathological analysis was obtained at the end of the experiment at day 95 p.i. from ECS- versus sham (control)-treated mice as shown on Figure 1C. In all graphs, the horizontal lines (and gray zone) represent the median value (±minima/maxima) of naïve, age-matched Biozzi mice. (A-E) Immune-fluorescent staining for CD3 in control EAE (A-B) and ECS-treated EAE (C-D) showed that ECS induced a 59% reduction in the total amount of CD3+ T cells in spinal cord white matter (WM, CD3+ cell quantification is provided as total number of cells per (uniformly cut) section (E). The white squares in A and C are enlarged in B and D respectively, arrowheads point to
CD3 positive cells.

(F-H) Immune-fluorescent staining for IBA1 in control EAE (F) and ECS-treated EAE (G) showed that ECS induced a 44% reduction in IBA1+ cells in SC WM (H). IBA1+ cell quantification is provided as number of cells per microscopic field.

(I-K) Immune-fluorescent staining for GFAP in control EAE (I) and ECS-treated EAE (J) showed that ECS induced a 35% reduction in GFAP+ cells in SC WM (H). GFAP+ cell quantification is provided as number of cells per microscopic field.

P values calculated with Student’s unpaired T test. Box-and-whisker plots show quartiles with median and minima and maxima at the bottom and top whiskers, respectively.
Figure 3.
Figure 3: ECS reduces demyelination and axonal injury in chronic EAE

Pathological evaluation was performed at the end of the experiment at day 95 p.i. from ECS- versus sham-treated mice as shown on Figure 1C. Horizontal lines (and gray zone) in Q and T represent median value (±minima/maxima) of naïve age-matched Biozzi mice.

(A-F) Gold-black staining for Myelin (A, C) and Bielschovsky staining for axons (B, D) in spinal cords of control EAE (A, B) and ECS-treated EAE mice (C, D). Black/white dashed line shows areas of myelin or axonal loss. ECS significantly reduced de-myelination (69% reduction, E) or axonal loss (97% reduction, F). Values are presented as percent of the average area of de-myelimation/axonal loss out of the total white matter area.

(G-N) Confirmation of histochemical analysis by immune-fluorescent staining for myelin and axonal markers. Serial adjacent sections were stained in uninjured and lesioned white matter of chronic EAE mice. In uninjured white matter Gold-black staining (G) correlated well with strong myelin basic protein (MBP) staining (H), and Bielschowsky staining (I) with neurofilament M (NF) staining (J). In lesions, loss of myelin (as found by Gold-black, K) correlated with marked reduction in MBP (L), and loss of axons (as found by Bielschowsky, M) with reduced NF staining (N).

(O-Q) Immune-fluorescent staining for APC in control EAE (O) and ECS-treated EAE (P) showed no difference in APC+ cells in SC WM (Q). APC+ cell quantification is provided as number of cells per microscopic field.

(R-T) Immune-fluorescent staining for NG2 in control EAE (R) and ECS-treated EAE (S) showed a 57% reduction in NG2+ cells in SC WM (Q). NG2+ cell quantification is provided as number of cells per microscopic field.

P values calculated with Student’s unpaired T test. Box-and-whisker plots show quartiles with median and minima and maxima at the bottom and top whiskers, respectively.
Figure 4: ECS attenuates EAE by a direct effect on the CNS.

EAE was induced in SJL mice by transfer of encephalitogenic T cells from donor mice to recipient mice.

(A-B) Donor mice received three consecutive ECS (n=7) or sham (control, n=7) treatments on days 8-10 p.i. 24 hours later lymph node cells were isolated from ECS-treated or the control group and injected into two groups of naïve mice (day 0). Disease severity and pattern were similar between the two recipient groups. Error bars = SD (A). No significant difference was found in the cumulative score between the mice which received encephalitogenic lymphocytes from the control or the ECS-
treated group (B). The experiment was repeated twice.

(C-D) Encephalitogenic lymphocytes from control or ECS treated mice were activated in vitro with PLP or ConA. Representative FACS image (from 3 independent experiments) of Brdu+ cells showing similar percentage of proliferating cells from ECS and sham-treated donor mice in response to PLP (C). FACS analysis of Brdu stained cells showed no significant difference in the percent of Brdu+ cells between the groups when not activated (naïve) or activated with PLP (quantification of C) or ConA (D). Three independent experiments were performed, Error bars = SEM.

(E-F) Recipient mice treated with three consecutive ECS (n=10 mice) or sham (n=11 mice) treatments, starting at day 8 post-transfer, upon first presentation of clinical signs. Disease severity and length was reduced among the ECS treated group. Error bars = SD (E). Cumulative score was significantly reduced in the recipient mice treated with ECS (F). The experiment was repeated twice. Box-and-whisker plots show quartiles with median and minima and maxima at the bottom and top whiskers, respectively. P values calculated with Student’s unpaired T test.
Figure 5: ECS does not affect the blood brain barrier permeability.

Fluorescence microscopy in white matter (WM, A-D) and gray matter (GM, E-H) showed no Biocytin-TMR in the spinal cord of naïve Biozzi ABH mice (n=3 mice, A, E), robust fluorescence in Biozzi ABH mice during acute EAE relapse (n=3 mice, B, F), and negligible fluorescence in sham-(control, n=3 mice, C, G) and ECS-treated (n=3 mice, D, H) spinal cords at day 38 p.i. (EAE remission), following 3-consecutive days treatment sessions. Biocytin-TMR fluorescence was measured as intensity optical density (IOD) and presented as percent of fluorescence during peak of acute relapse (I). Error bars = SEM.
Figure 6: ECS decreases microglial toxicity.

(A-G) Immune-fluorescent staining for IBA1 and iNOS in SC sections of sham (control) - (A) and
ECS-treated (B) Biozzi ABH EAE mice at day 95 p.i. ECS significantly reduced the number of IBA1+/iNOS+ cells in SC WM by 40%. Box-and-whisker plot shows quartiles with median and minima and maxima at the bottom and top whiskers, respectively. (C). Single color panels of IBA1 and iNOS are shown for image A (D and E, respectively), and for image B (F and G, respectively). (H-O) CD11b+ cells were isolated at day 38 p.i., 24 hours after the last of three ECS treatments (performed on days 35-37 p.i.) and evaluated by RT-PCR, FACS analysis and ELISA assays. Experiments were repeated three or four independent times. (H-K) mRNA levels of iNos (H), Il2 (I), Cxcl9 (J) and Il1b (K) were reduced in CD11b+ cells isolated from ECS- as compared to sham (control) -treated Biozzi EAE mice. In all graphs, the horizontal lines (and gray zone) represent the RQ mean value (±RQ min/max) of naïve, age-matched Biozzi mice. (L) FACS analysis of IBA1 expression showed marked reduction in IBA1 expression (and specifically in IBA1\textsuperscript{high} cells) in CD11b+ cells, isolated from ECS-treated spinal cords, as compared to sham (control)-treated mice (representative image). (M-O) Levels of NO (M) and ROS (N) production were decreased in isolated CD11b+ cells from ECS- as compared to control EAE mice, measured by ELISA reader in response to Griess Reagent or DCFDA respectively. FACS analysis (O) of intracellular ROS (response to DCFDA) in the isolated CD11b+ cells from EAE mice showed two cell populations: low and high ROS producing cells (representative image). ECS reduced the percent of high ROS producing CD11b+ cells. P value calculated with students unpaired T test. Error bars- RQ min/max in H-K and SEM in M-O.
Figure 7: ECS reduces oxidative injury in spinal cords of ECS-treated EAE mice.

(A-B) Immune-fluorescent staining for MDA in SC sections of EAE Biozzi mice (day 95 p.i.). ECS reduced the number of oxidative injured MDA+ cells (shown as fraction of total cells) within the white matter (WM) and gray matter (GM) of the SC (C). In A-B dashed line demarcates the WM/GM border, arrows point at MDA+ cell in the WM, chevron arrows point at MDA+ cell in the GM.

(D) Double staining for NeuN (marker for neurons) and MDA showing localization of MDA staining in neurons in the GM of EAE mice, marked by arrowheads.

(E) Double staining for APC (marker for oligodendrocytes) and MDA showing localization of MDA staining in oligodendrocytes in the WM of EAE mice, marked by arrowheads.
Box-and-whisker plot shows quartiles with median and minima and maxima at the bottom and top whiskers, respectively. P values calculated with Student’s unpaired T test.