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P.)Abstract: The development of therapies for multiple sclerosis (MS) targeting CNS inflammation and protecting neurons is imperative. Here we demonstrate that the neuroprotective neurosteroid derivative BNN27, which is not metabolized to estrogens or androgens, reversed established acute, as well as relapsing-remitting experimental autoimmune encephalomyelitis (EAE) through a dramatic suppression of pathogenic TH1 and TH17 responses, accompanied by a down-regulation of CCR6 expression in the latter cells. In addition, BNN27 administration induced CD4+ T cells suppressive in vitro and in vivo in an IL-10-dependent manner. Dehydroepiandrosterone (DHEA), the natural homologue of BNN27, exerted similar IL-10-dependent immunosuppressive effects on established EAE. BNN27 and DHEA also inhibited human TH17 polarization and suppressed human TH17 responses from MS patients. The immune-modulatory effects of BNN27 and DHEA were largely mediated by nerve growth factor receptors. Our findings suggest that BNN27 represents a new class of therapeutic agents for MS. Main Text: Multiple sclerosis (MS) is a devastating autoimmune disease of the central nervous system (CNS). Despite recent significant milestones in MS treatment, there is a great need for novel improved therapeutic approaches, especially combining neuroprotection with immunomodulation. MS pathogenesis has been widely studied using the mouse model experimental autoimmune encephalomyelitis (EAE). EAE develops when brain and spinal cord are infiltrated by T lymphocytes autoreactive against CNS antigens, causing activation of glia, demyelination and neuronal degeneration . The detrimental inflammatory response in EAE is mediated by two different lineages of T helper lymphocytes characterized by the production of either interferon-γ (IFN-γ; TH1 cells) or interleukin-17 (IL-17; TH17 cells) . Increasing TH17 to TH1 ratio results in higher EAE severity . Both TH1 and TH17 cells have been demonstrated in brain lesions of MS patients . Suppressive regulatory T (Treg) cells are important in limiting autoreactive TH1 and TH17 responses in the CNS , and MS has been correlated with several defects in Treg cell function . Thus, therapies for MS targeting the induction of functional regulatory cells and suppression of pathogenic autoreactive T cell responses are of high priority. In parallel, the ability of these same treatments to rescue neurons from apoptosis, minimizing brain atrophy would significantly add to their efficacy. Administration of dehydroepiandrosterone (DHEA), the most abundant adrenal steroid in humans , has been shown to exert beneficial effects in EAE . However its mechanism of action, especially on the immune cells, is not understood. DHEA is additionally produced by neurons and glia (neurosteroid), affecting multiple processes in the brain, including neuronal survival and neurogenesis during development and in aging . In peripheral tissues, DHEA is converted into androgens and/or estrogens, which also have variable immunomodulatory functions accompanied by several side effects . We have recently synthesized a DHEA derivative, BNN27 [(20R)-3β, 21-dihydroxy-17α, 20-epoxy-5-pregnene] (Fig. 1A) , deprived of estrogenic or androgenic actions, which possesses strong neuroprotective properties with an EC50 at nanomolar levels . Here, we investigated BNN27 as a potential immunomodulatory agent for established CNS autoimmunity. We administered BNN27 into symptomatic (1-2 clinical score) mice with MOG35-55-induced EAE and they became almost free of paralysis within 2-3 days of treatment (mean maximum scores; 0. 142857 ± 0. 154303 for BNN27 group, versus 3 ± 0. 408248 for PBS group, Fig. 1B and fig. S1A). We have also tested DHEA, the natural homologue to BNN27. In addition, as DHEA was reported to attenuate EAE when administered at disease induction, and well before clinical onset , it would be important to determine whether it would be beneficial to symptomatic mice. DHEA administration suppressed EAE, while control-treated mice presented severe symptoms (Fig. 1B and fig. S1A). CNS mononuclear cells from BNN27- and DHEA-treated mice were significantly lower in comparison to PBS-treated mice (fig. S1B). Spinal cord sections from BNN27- and DHEA-treated mice showed decreased degree of inflammation (Fig. 1C), a significant decrease in T cell infiltration (Fig. 1, D and E), as well as decreased microglial activation (fig. S1C). Whereas control-treated mice displayed substantial demyelination, BNN27- and DHEA-treated mice had intact spinal cords (Fig. 1E). MOG35-55 –specific responses of BNN27- or DHEA-treated mice were significantly suppressed (fig. S2A), characterized by decreased levels of IL-17, IFN-γ, osteopontin (Opn) and TNF-α (Fig. 1F and fig. S2B). This was also accompanied by a significant reduction in the numbers of both TH17 (Fig. 1G) and TH1 cells (fig. S2C) in the draining lymph nodes (DLNs). The gene expression of the orphan nuclear receptor Rorc, master transcription factor of TH17 cells, as well as of the Il17 gene, were down-regulated in DLN cells from BNN27-treated EAE mice (fig. S2D). These findings suggest that BNN27, and DHEA, suppress pathogenic effector TH responses, effectively controlling ongoing CNS autoimmunity. MOG35-55–specific responses of BNN27- or DHEA-treated mice were characterized by significantly increased production of the immunoregulatory cytokine IL-10 (Fig. 1F and fig. S2B) along with increased frequency of IL-10-producing CD4+ T cells, as well as of IL-10/IL-17-producing CD4+ T cells in the DLNs and the CNS (Fig. 1G and fig. S2E). Both IL-10+ and IL-10/IL-17+ CD4+ T cell subsets have been demonstrated to be suppressive in EAE . We confirmed the up-regulation in the percentage and numbers of IL-10-producing CD4+ T cells using Il10gfp (Il10tm1. 1Karp) mice that we treated with BNN27 after EAE clinical onset (fig. S3A). Isolated CNS mononuclear cells from BNN27-treated mice had increased expression of the Il10 gene (fig. S3A), as compared to PBS control-treated mice. In addition, CD4+ T cells from BNN27-treated mice had increased expression of the Il10ra gene (fig. S3A), indicating responsiveness to immune suppression by IL-10 . The secreted levels of TGF-β1 were not altered (fig. S2B). Notwithstanding its immune-regulatory functions, TGF-β1 can lead to TH17- or TH9-mediated pathology, depending on the cytokine milieu . Furthermore, the percentage of CD4+ CD25+ Foxp3+ T regulatory cells was elevated in DLNs of BNN27-treated EAE mice, as compared to PBS-treated mice (fig. S3B). Similar immunomodulatory effects of BNN27 and DHEA in the course of EAE were also observed in mice euthanized at an earlier time point during disease peak (approximately day 17) (fig. S4, A and B). BNN27 and DHEA were equally suppressive even when administered at lower daily doses (≥ 1mg/day, data not shown). Overall, BNN27 and DHEA appear to suppress pathogenic TH1 and TH17 responses while increasing numbers of T cells with regulatory phenotype. CCR6 is a chemokine receptor essential for the function of effector TH17 cells, as well as for their entry into the CNS and the establishment of EAE . Analysis of CCR6 expression in DLN TH17 cells from BNN27-, DHEA- or PBS-treated EAE mice at early disease stages showed that BNN27- and DHEA-treated mice had significantly lower CCR6 expression on TH17 cells, compared to PBS-treated EAE mice (Fig. 1H and fig. S3C), suggesting that BNN27 and DHEA affect the infiltration of inflammatory TH17 cells into the CNS. EAE can be transferred to recipients by autoreactive CD4+ T cells . Rag1-/- mice that received CD4+ T cells from BNN27-treated EAE donors had significantly milder paralysis and delayed disease onset (Fig. 2A), as compared to Rag1-/- mice that received equal numbers of CD4+ T cells from PBS-treated donors. Suppressed disease score was accompanied by significantly increased production of IL-10 and decreased production of IL-17 and IFN-γ (Fig. 2B). CD4+ T cells from DHEA-treated mice had reduced pathogenic potential upon transfer as well (fig. S5). These findings suggest that effector CD4+ T cells from BNN27- or DHEA-treated EAE mice remain significantly less pathogenic even after transfer into lymphopenic, untreated recipients. We also tested whether BNN27 had therapeutic effects on symptomatic SJL mice that suffered from relapsing-remitting EAE induced by immunization with proteolipid protein peptide PLP139-151, a different to MOG autoantigen. Therapeutic administration of BNN27 significantly suppressed disease (Fig. 2C and fig. S6A), decreasing levels of IFN-γ and IL-17 in response to PLP139-151 peptide (Fig. 2D) and reducing numbers of isolated CNS mononuclear cells (fig. S6B), as well as numbers of DLN TH17 and TH1 cells (fig. S6C) and CNS TH17 cells (Fig. 2E). Numbers of CCR6+ TH17 cells were also decreased (fig. S6C) following BNN27 treatment. The gene expression of Rorc, was down-regulated in DLN cells from BNN27-treated EAE mice (fig. S6D). In parallel, IL-10 levels, as well as spinal cord IL-10- and IL-10/IL-17-producing CD4+ T cells were significantly increased in BNN27-treated EAE mice (Fig. 2, D and E). Similar to BNN27, DHEA administration had beneficial effects on EAE of SJL mice (fig. S7, A to E). Thus, BNN27 and DHEA effectively suppress ongoing autoreactive responses independently of the inducing autoantigen and the type of EAE. Of note, disease remained suppressed, even when we re-challenged DHEA- (fig. S8A) and BNN27 -treated (data not shown) mice with MOG35-55/IFA to induce relapse. MOG35-55-specific secretion of IFN-γ and IL-17 remained significantly lowered, while IL-10 secretion remained significantly elevated (fig. S8B). When we re-injected DHEA- (fig. S8C) and BNN27-treated (data not shown) mice with pertussis toxin to facilitate blood-brain barrier breakdown, their clinical phenotype, as well as MOG35-55–specific IFN-γ and IL-17 secretion remained significantly suppressed (fig. S8D). Again, IL-10 secretion in DHEA-treated EAE mice remained significantly up-regulated (fig. S8D). Thus, certain pathogenic triggers cannot cancel disease suppression conferred by BNN27 or DHEA. The suppressive effects of BNN27 or DHEA were long-lasting; symptomatic EAE mice treated with BNN27 or DHEA for 15 days remained free of paralysis for 50 more days with no treatment (Fig. 2F). To test whether BNN27-mediated suppression was targeted mainly to ongoing autoreactive responses, we immunized BNN27- and DHEA-treated EAE mice to an irrelevant antigen (ovalbumin). This resulted in a successful TH1 immune response to this new antigen (fig. S8E, F). We observed a similar enhanced TH1 immune response when we challenged BNN27- and DHEA-treated mice with inactivated lentivirus (data not shown). These findings suggest that BNN27 and DHEA act effectively against the myelin-specific ongoing autoreactive responses without dampening the protective responses induced by pathogen-mimicking agents, consistent also with previous studies showing that DHEA enhances anti-microbial immunity . Similarly, administration of BNN27 or DHEA, starting at the time of EAE induction with MOG35-55 in CFA (containing inactivated M. tuberculosis) resulted in significantly enhanced TH1 responses (fig. S9B). However, disease severity was milder (fig. S9A), accompanied by decreased CNS inflammation (fig. S9D), as well as decreased IL-17/IFN-γ ratio (fig. S9C). Overall, it appears that BNN27 and DHEA act more effectively when the autoimmune response is established, which is of clinical importance. This could be attributed to differential regulation of the entry of inflammatory TH1 and TH17 cells into the CNS . We have also tested the ability of BNN27 to control established EAE in 2D2 TCR Tg mice that are greatly susceptible, as they bear high numbers of MOG35-55–specific CD4+ T cells . Administration of BNN27 in symptomatic EAE 2D2 TCR Tg mice resulted in significantly milder symptoms, as compared to PBS-treated mice (mean maximum scores; 2. 25± 0. 25 for BNN27 group, versus 3. 75±0. 25 for PBS group, Fig. 3A and Suppl. Video). MOG35-55–specific responses of BNN27- treated 2D2 TCR Tg mice were significantly suppressed (fig. S10A), characterized by decreased IL-17, IFN-γ and IL-6 secretion (Fig. 3B and fig. S10B), while IL-10 was up-regulated (Fig. 3B and fig. S10B). Expression levels of Rorc and Ccr6 were also down-regulated in DLN 2D2 CD4+ T cells from BNN27-treated mice, (Fig. 3C and fig. S10B), compared to control. Therefore, BNN27 was also effective in suppressing EAE and TH17 responses in highly predisposed 2D2 TCR Tg mice. BNN27 was also effective in controlling the activation of 2D2 TCR Tg T cells in vitro. Culture of DLN cells from 2D2 TCR Tg mice with MOG35-55 in the presence of 100 nM BNN27 or DHEA also resulted in a remarkable decrease of the percentages of IL-17+CD4+ T cells, while the percentage of IL-10+IL-17+ T cells among TH17 cells was significantly increased (Fig. 3D). Additionally, expression levels of Il17, Il6, Ccr6 and Rorc were down-regulated in 2D2 CD4+ T cells cultured in the presence of BNN27 and DHEA (fig. S10C). Both agents exerted similar effects in ex vivo re-stimulated DLN CD4+ T cells from mice with EAE (data not shown). We also observed direct effects of BNN27 on purified CD4+ T cells, as BNN27 suppressed the anti-CD3/anti-CD28 –driven CD4+ T cell proliferation (fig. S10D). To test the effects of BNN27 and DHEA on ongoing TH17 responses, we re-stimulated DLN cells from mice with EAE (10 days after immunization) with MOG35-55 in the presence of IL-6, TGF-β and IL-23, known to maintain TH17 responses, in the presence of BNN27 or DHEA or RPMI, for 7 days . Both steroids decreased the percentages of IL-17+ CD4+ T cells (Fig. 3E) and IL-17 secretion (Fig. 3G), concomitant with increased percentages of IL-10+ IL-17+ CD4+ T cells among TH17 cells (fig. S10E) and elevated IL-10 secretion (Fig. 3F). Both steroids did not affect the numbers of 7AAD-positive CD4+ T cells (data not shown). BNN27 and DHEA were ineffective in suppressing IL-17 secretion and IL17+ CD4+ T cell percentages when CD4+ T cells were Il10-deficient (Fig. 3, E and G). Furthermore, sorted IL-10- CD4+ T cells from Il10gfp EAE mice that were stimulated in TH17 maintenance conditions (as in Fig. 3E) in the presence of BNN27 were unable to revert to an IL-10-producing population (fig. S11A). In this setting, IL-17+ CD4+ T cells were not suppressed by BNN27 (fig. S11A). However, in parallel cultures of sorted IL-10+ CD4+ T cells, we observed expansion of IL-10+ CD4+ T cells in the presence of BNN27 (fig. S11B). It appears that the suppressive function of BNN27 relies on the presence of IL-10-producing cells. In addition, these findings indicate that the beneficial effects of BNN27 in EAE involve direct suppressive effects on immune cells. Treatment of EAE mice with BNN27 results in the induction of CD4+ T cells with regulatory phenotype that could potentially suppress autoreactive responses. CD4+ T cells from BNN27-treated EAE mice were co-transferred into lymphopenic Rag1-/- recipients, in combination with CFSE-labeled effector CD4+ T cells from donors with EAE. Rag1-/- recipients adoptively co-transferred with CD4+ T cells from BNN27-treated EAE donors had significantly decreased numbers of proliferating CFSE-labeled effector CD4+ T cells in the DLNs, delayed disease onset and significantly reduced disease clinical score compared to control recipients (Fig. 4, A and B). We have also examined whether TH cells from BNN27- and DHEA–treated EAE mice could suppress ongoing EAE upon adoptive transfer into symptomatic wild type mice. Transfer of CD3+ or CD4+ T cells from BNN27- and DHEA-treated mice suppressed the ongoing disease of recipients (Fig. 4, C and D and fig. S12A). Significantly reduced ratios of IL-17+/IFN-γ+ T cells in the CNS (fig. S12B), and of IL-17/IFN-γ levels secreted by DLN cells (fig. S12C) were found in recipients of CD3+ cells from BNN27 or DHEA- treated EAE donors, compared to control recipients. Significant suppression of the disease was also found in recipient mice with ongoing EAE adoptively transferred with DLN (fig. S13) and with CD11c+ cells from BNN27- or DHEA- treated EAE mice (Fig. 4E). In fact, adoptive transfer of CD11c+ cells from BNN27- or DHEA- treated mice into recipients with ongoing EAE significantly decreased the level of IL-17, IFN-γ (fig. S14A) and the numbers of CCR6+ TH17 cells in DLNs (fig. S14B). It is possible that Treg cells induced by BNN27 educate DCs to become tolerogenic . However, co-culture of BNN27-pre-treated naïve CD11c+ cells together with CD4+ T cells from day 10- EAE mice resulted in a significant up-regulation of IL-10 secretion by the CD4+ T cells (data not shown), indicating that BNN27 has also a direct effect on DCs. Taken together, these findings suggest that treatment of BNN27 or DHEA induces, besides functional regulatory T cells, additional immune cell populations with suppressing properties of ongoing EAE in vivo. To elucidate the role of IL-10 in BNN27- and DHEA-mediated EAE suppression, we tested the ability of these neurosteroids to control disease in Il10-/- EAE mice. Both BNN27 and DHEA were ineffective to suppress the disease in the absence of IL-10 (Fig. 4F). In contrast to wild type EAE mice, BNN27 and DHEA did not suppress the secretion of IL-17 in Il10-/- EAE mice (Fig. 4G). We have also tested the ability of neutralizing antibodies against IL-10 to reverse the suppressive effects of BNN27 or DHEA in wild type EAE mice. Antibody-neutralization of IL-10 blunted the suppressive effects of BNN27 and DHEA, increasing the severity of EAE to levels observed in PBS-treated control EAE mice (fig. S15, A and B). CD4+ T cells from BNN27-treated Il10-/- EAE mice preserved their pathogenicity after transfer to Rag1-/- recipients (fig. S15C). In vitro, 2D2 TCR CD4+ T cells from BNN27-treated EAE mice were stronger suppressors of CFSE-labeled 2D2 TCR CD4+ T effector cells, than 2D2 TCR CD4+ T cells from PBS-treated EAE mice (Fig. 4H). Blockade of IL-10 reversed this suppression (Fig. 4H). We conclude that IL-10 induction mediates, to a great extent, the suppressive effects of the BNN27 treatment in EAE. We have also tested whether BNN27 and DHEA affect the differentiation of naïve human CD4+ T cells towards TH17. BNN27 at 100 nM in combination with a mixture of TGF-β, IL-6, IL-21 and IL-1β significantly decreased the secretion of IL-17 in human CD4+ T cells in culture (Fig. 4I), reducing also the number of IL-17+ CD4+ T cells (Fig. 4J). It is of note that BNN27 decreased the numbers of both TH1 and TH17 cells, in the absence of polarizing factors (Fig. 4J). DHEA had similar immunomodulatory effects (fig. S16, A-C). Notably, in the same cell cultures, DHEA increased the number of human IL-10+ CD4+ T cells (fig. S16C), compared to CD4+ T cells, stimulated with the mixture of TGF-β, IL-6, IL-21 and rIL-1β alone. We have also assessed the effects of BNN27 and DHEA in T cells, isolated from eight MS patients with RR-MS (Table S1). BNN27 and DHEA significantly decreased the release of IL-17, IFN-γ and IL-4 cytokines, while IL-10 and TNF-α remained unaffected (Fig. 4K). In MS, IL-4 secretion inhibits Treg cell induction . Our data suggest that in addition to EAE mice, BNN27 and DHEA may limit human autoreactive TH17 responses in MS patients by interfering with both TH17 polarization and ongoing TH17 responses. We have recently shown that DHEA binds with high affinity to NGF receptors . Indeed, DHEA exerts at least part of its neuroprotective actions by directly interacting with TrkA and p75NTR receptors (KD: 5-10 nM), efficiently inducing TrkA phosphorylation at tyrosine-490, and p75NTR interaction with its effector proteins RIP2 and TRAF6 . Based on these observations, in the present study we tested the ability of BNN27 to bind to NGF receptors. BNN27 effectively competed binding of [3H]-DHEA to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA or p75NTR receptors with Ki at nanomolar levels (Fig. 4L). Immune cells, such as T cells and macrophages strongly express NGF receptors ,. BNN27 at 100 nM effectively induced the phosphorylation of TrkA at tyrosine 490 in isolated CD4+ T cells from EAE mice (Fig. 4M). BNN27 at 100 nM facilitated the association of p75NTR receptor to its effector protein RIP2 in mouse CD4+ T cells in culture (Fig. 4M). Consistently, in cultures of DLN cells from EAE-induced mice, a synthetic inhibitor of TrkA phosphorylation and an antibody against p75 abrogated the induction of IL-10 by BNN27 or DHEA (Fig. 4N and fig. S17A). In addition, the suppressive effects of BNN27 and DHEA on Ccr6 gene expression (as in fig. S10C), were no longer observed when we cultured CD4+ T cells in the presence of a synthetic inhibitor of TrkA receptor (fig. S17B). In the same cultures, TrkA inhibition, as well as blocking of p75 receptor reversed the suppression of IL-17 secretion (data not shown). Our findings suggest that NGF receptors contribute to the suppressive effects of BNN27 and DHEA. Several studies demonstrate the involvement of the NGF system in EAE and MS. NGF was shown to protect marmosets against EAE by down-regulating the production of IFN-γ by CNS-infiltrating T cells, and up-regulating the production of IL-10 by glial cells in inflammatory lesions . NGF administration suppressed EAE , while neutralization of NGF or deficiency in p75NTR receptor expression exacerbated EAE in mice . Interestingly, enhanced expression of NGF receptors was reported in EAE and MS lesions, most probably as a rescue response . Disease up-regulation of NGF receptors in the EAE and MS lesions may enhance the efficacy of BNN27 in suppressing neuroinflammation. A DHEA metabolite towards its conversion to androgens and estrogens, 5-androsten-3β, 17β-diol (ADIOL), exerts anti-inflammatory effects on microglia and astrocytes in EAE mice, acting via estrogen receptor beta (ERβ) . Our experiments showed that both DHEA and BNN27 dampened IL-6 secretion, as well as iNOS expression, compared to control-treated primary microglia and BV2 cells (fig. S17, C and D). Also, Il10 expression was elevated in BV2 cultured in the presence of DHEA and BNN27, although it was not statistically significant (fig. S17D). Both DHEA and its metabolite ADIOL, were shown to bind, the former with low and the latter with high affinity, to estrogen receptor beta (ERβ Ki: 200 nM and 1 nM respectively) . In contrast to DHEA and ADIOL, BNN27 was unable to compete binding of [3H]-Estradiol to cytosolic preparations isolated from HEK293 cells transfected with the cDNA of ERbeta receptors (fig. S18A). ADIOL was unable to reverse binding of [3H]-DHEA to membranes isolated from HEK293TrkA transfectants while effectively competing its binding to membranes from HEK293p75NTR transfectants (Ki: 1. 05+x nM) (fig. S18B). Moreover, ADIOL at 100 nM facilitated, within 20 min, the association of p75NTR receptor to its effector protein RIP2 in mouse CD4+ T cells in culture (Fig. 4N). Our findings suggest that, in addition to ERβ receptors, p75NTR receptors may also contribute to the previously observed suppressive effects of ADIOL in EAE mice. Glial cells strongly express p75NTR receptors, which hold a central role in the control of their differentiation, migration, proliferation and activation . For example, it is possible that ADIOL suppresses microglia activation via ERβ receptors, while affecting astrocytes through p75NTR receptors. Our data also point to IL-10-mediated suppression of ongoing autoimmune responses by DHEA. DHEA and ADIOL activate ERβ receptors , are intermediates in estrogen and androgen biosynthesis , and their administration not only impairs the endocrine system long-term, but also poses a risk for estrogen- and androgen- dependent tumors, particularly in genetically predisposed patients (Miller et al. Steroids, 2013). Immunomodulatory synthetic derivatives, which lack estrogenic and androgenic properties, are far more suitable for treatment of chronic inflammatory conditions. Here we show that the derivative BNN27, a neuroprotective microneurotrophin , is a strong inducer of IL-10-mediated immune regulation and successfully suppresses pathogenic autoreactive responses and disease. BNN27 can serve as prime molecule for development of specific immunotherapies that induce IL-10-expressing T cells, pivotal in the regulation of immune responses and in the maintenance of immunological tolerance . Moreover, our findings add to insights on the crosstalk between the immune system and neurotrophic components .

## References and Notes:

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TC is supported by the Deutsche Forschungsgemeinschaft, the European Research Council and the Novartis Foundation for Therapeutical Research. CT is supported by European and National funds under " Aristeia" grant (EPEDBM-2071). Author contributions: M. A. designed experiments, performed animal studies, tissue-culture experiments, flow cytometry, analyzed data, generated figures and wrote the manuscript with V. P.. E. K. performed animal studies, cell studies and flow cytometry analysis and provided ideas. I. L. performed confocal microscopy studies. D. C. M. S. designed primers, performed and analyzed RT-PCR assays. N. P. assisted with animal studies and suppression assay design and analysis. D. K. and A. M. performed and analyzed MS subject studies. V. I. A. performed experiments on microglia. T. C. and C. T. supervised and analyzed microglia experiments. I. C. provided ideas and assisted with the design and analysis of experiments. ND and PP were responsible for MS patient selection, follow up, consent form approval, and collection of blood samples. H. C. provided ideas and critically edited the manuscript. A. G. provided ideas, and assisted with experimental design and writing of the manuscript. V. P. provided ideas, designed experiments, analyzed data, supervised the study and wrote the manuscript with M. A. Fig. 1. BNN27 treats acute EAE. (A) Structure of DHEA and BNN27. (B) Clinical EAE scores (means ± s. e. m.) over time. n = 7-8 mice per group. Data are representative of three independent experiments. \*\*\* P = 0. 0001 for DHEA versus PBS group, \*\*\* P < 0. 0001 for BNN27 versus PBS group. (C) Sections of spinal cord were obtained 30 days after immunization and stained with H&E to assess inflammation. \* P = 0. 0133, \*\*\* P = 0. 0009, Scale bars, 50μm. (D) Histology of spinal cords sections. Sections were obtained 30 days after immunization and stained with anti-CD3 and anti-GFAP to assess inflammation and migrating astrocytes. Scale bars, 200μm for the left panel and 50μm for the right panel. (E) Sections of spinal cord were obtained 30 days after immunization and stained with Hoechst nuclear staining and anti-MBP to assess myelin depletion. An area of ~5mm of the spinal cord at thoracic-lumbar level from 3 longitudinal sections (separated at least 300μm), from 3 different animals in each group, were analyzed to quantify the area of infiltration in white mater. Scale bars, 100μm. \*\* P = 0. 0037 (unpaired Student’s t test) for DHEA versus PBS group, \*\* P = 0. 0025 for BNN27 versus PBS group. BNN27 suppresses effector TH responses and induces T regulatory cell subsets. (F) IL-17 (\*\*\* P = 0. 0003 for DHEA versus PBS group, \*\*\* P = 0. 0007 for BNN27 versus PBS group, \*\* P = 0. 0011) and IL-10 (\*\*\*P = 0. 0009 for DHEA versus PBS group, \*\*\* P < 0. 0001 for BNN27 versus PBS group, \*\* P = 0. 0061) levels were measured by ELISA in the supernatants of DLN cells stimulated ex vivo with MOG35-55 peptide from EAE-induced mice and treated with BNN27 or DHEA or PBS. (G) Numbers of DLN IL-17+IL-10-CD4+ T cells (\*\* P = 0. 0055, \*\*\* P = 0. 0006), IL-10+CD4+ T cells (\*\* P = 0. 0027 for DHEA versus PBS group, \*\* P = 0. 0015 for BNN27 versus PBS group) and numbers of IL-17+IL-10+CD4+ T cells, \* P = 0. 024. One representative of three independent experiments. (H) Percentages and numbers of DLN CCR6+IL-17+CD4+ T cells. \*\* P = 0. 0071 for DHEA versus PBS group, \*\* P = 0. 001 for BNN27 versus PBS group. Unpaired Student’s t test for all statistical analysis. Fig. 2. CD4+ T cells from BNN27-treated mice are less pathogenic and BNN27 treats relapsing-remitting EAE. (A) Clinical EAE scores (means ± s. e. m.) over time (on day 22, \*P = 0. 0370) of C57BL/6 Rag1-/- mice adoptively transferred with purified CD4+ T cells from C57BL/6 EAE mice treated with BNN27 or PBS for 7 days. n = 4-5 mice per group. One representative of three independent experiments. (B) IL-10 (\*\*\* P < 0. 0001), IL-17 and IFN-γ (\*\*\* P = 0. 0004) levels were measured in the supernatants of DLN cells from mice in 2a. (C) Clinical EAE scores of SJL mice (means ± s. e. m.) over time (on day 18; \*\*P = 0. 0014, on day 25 \*P = 0. 0395). n = 5-6 mice per group. One representative of two independent experiments. (D) IFN-γ (\*\*\* P < 0. 0001), IL-17 (\*\* P = 0. 0069) and IL-10 (\*\* P = 0. 0031) levels were measured by ELISA in the supernatants of DLN cells stimulated ex vivo with PLP139-151 peptide from mice in 2c. (E) Percentages of spinal cord IL-10+CD4+ T and IL-17+IL-10+CD4+ T cells. Unpaired Student’s t test for all statistical analysis. BNN27 and DHEA suppressive effects are stable. (F) Clinical EAE scores (means ± s. e. m.) over time of BNN27-, DHEA- or PBS-treated EAE mice for 15 days and then left untreated for 45 days. \* P = 0. 0498 on day 21, \*\* P = 0. 0019 on day 26, \*\*\* P = 0. 0019 on day 30. n = 5 mice per group. One representative of two independent experiments. Unpaired Student’s t test for all statistical analysis. Fig. 3. BNN27 suppresses EAE and TH17 responses in MOG35-55–specific T-cell receptor transgenic mice, while induces IL-10. (A) Clinical EAE scores of MOG35-55–specific T-cell receptor transgenic (2D2TCR Tg) mice (means ± s. e. m.) over time (on day 14;\* P = 0. 017, on day 15 \*\* P = 0. 0054). n = 5-6 mice per group. One representative of three independent experiments. (B) IL-17 (\*\*\* P = 0. 0001), IFN-γ (\*\* P = 0. 0027), IL-6 (\*\*\* P <0. 0001) and IL-10 (\* P = 0. 038) levels were measured in the supernatants of DLN cells from 2D2 TCR Tg mice, re-stimulated ex vivo with MOG35-55 peptide. (C) mRNA expression of Rorc in CD4+ T cells isolated from 2D2 DLN cells. \* P = 0. 0284. (D) Percentages of IL-17+ on CD4+ T cells and of IL-10+ on IL-17+ CD4+ T cells in cultures of DLN 2D2 cells, stimulated ex vivo with MOG35-55 peptide and treated with 100nM BNN27, or 100nM DHEA or RPMI for 4 days. One representative of two independent experiments. (E) Percentages of IL-17+ on CD4+ T cells from day 10- Il10+/+ and Il10-/- EAE mice, cultured for 7 days with TGF-β, IL-6 and IL-23, in the presence of 100nM BNN27 or 100nM DHEA or RPMI. One representative of two independent experiments. (F) IL-10 (\*\*\* P = 0. 0008, \*\*\* P = 0. 0006) levels were measured in the supernatants of DLN cells cultured as in 3E. (G) IL-17 (\* P = 0. 0032, \*\* P < 0. 0001, \*\*\* P = 0. 0018, \*\*\*\* P = 0. 0033, \*\*\*\*\* P = 0. 0062) levels were measured in the supernatants of DLN cells cultured as in 3E. Unpaired Student’s t test for all statistical analysis. Fig. 4. Immune cells from BNN27- treated EAE mice are suppressive in vivo. (A) Clinical EAE scores (means ± s. e. m.) over time of C57BL/6 Rag1-/- EAE recipient mice adoptively co-transferred with purified CD4+ T cells from BNN27- or PBS- treated EAE mice together with purified CFSE-labeled CD4+ T cells from day 10- EAE mice. \*\* P = 0. 0074 on day 21, \*\*\* P < 0. 0001 on days 23-25. n = 4-5 mice per group. One representative of two independent experiments. (B) Numbers of CFSE+ proliferating cells in the DLNs of Rag1-/- EAE recipients, 8 days after the adoptive co-transfer. \* P = 0. 014. Data are expressed as mean ± s. e. m., two independent experiments. (C) Clinical EAE scores (means ± s. e. m.) over time of B6 EAE recipient mice adoptively transferred with CD3+ cells from BNN27-, DHEA- or PBS- treated EAE donors for 7 days (On days 20-22; \* P = 0. 0321 for DHEA versus PBS group, \*\* P = 0. 0054 for BNN27 versus PBS group). n = 4-5 mice per group. One representative of two independent experiments . (D) Clinical EAE scores (means ± s. e. m.) over time of B6 EAE recipient mice adoptively transferred with purified CD4+ T cells from BNN27- or DHEA- or PBS treated EAE mice, for 7 days. \*\* P = 0. 0071 on day 21 for DHEA versus PBS group, \* P = 0. 0474 on day 25 for DHEA versus PBS group, \*\* P = 0. 0071 on day 27 for DHEA versus PBS group, \* P = 0. 0151 for BNN27 versus PBS group. n = 4-5 mice per group. One representative of two independent experiments. (E) Clinical EAE scores (means ± s. e. m.) over time of B6 EAE recipient mice adoptively transferred with purified CD11c+ cells from BNN27-, DHEA- , or PBS-treated EAE mice for 7 days. n = 4-5 mice per group. \* P = 0. 0267 on day 16 for DHEA versus PBS group, \*\* P = 0. 0015 on day 18, for DHEA versus PBS group, \*\* P = 0. 0023 on day 20 for DHEA versus PBS group. One representative of two independent experiments. Unpaired Student’s t test for all statistical analysis. BNN27-mdiated disease suppression is IL-10-dependent. (F) Clinical EAE scores (means ± s. e. m.) over time of Il10+/+ and Il10-/- mice treated as described in Fig. 1B. \*\* P = 0. 0085 for DHEA Il10+/+ versus PBS Il0+/+ group, \*\* P = 0. 0085 for BNN27 Il10+/+ versus PBS Il10+/+ group. n = 4-5 mice per group, representative of three independent experiments. (G) IL-17 levels were measured in the supernatants of DLN cells stimulated ex vivo with MOG35-55 peptide. \* P = 0. 0273 for DHEA Il10+/+ versus PBS Il0+/+ group, \* P = 0. 03 for BNN27 Il10+/+ versus PBS Il10+/+ group, \* P = 0. 0287 for DHEA Il10-/- versus PBS Il10-/- group, \*\* P = 0. 0054 for DHEA Il10+/+ versus DHEA Il0-/- group, \* P = 0. 0187 for BNN27 Il10+/+ versus BNN27 Il0-/- group. (H) Numbers of CFSE+ proliferating cells from in vitro suppression assay with purified CD4+ T cells from BNN27- or PBS- treated 2D2 TCR Tg EAE mice together with sorted naïve CFSE-labeled CD4+ T responder cells from 2D2 TCR Tg mice and mitomycin-C-treated splenocytes as APCs. 10μg/ml of anti-IL-10 was added in the indicated groups. \* P = 0. 0136, \*\* P = 0. 0363, \*\*\* P = 0. 0041. Data are expressed as mean ± s. e. m., two independent experiments. (I) IL-17 levels were measured in the supernatants of human CD4+ T cells stimulated ex vivo for 7 days with TGF-β, IL-6, IL-21 and IL-1β in the presence of 100nM BNN27 or RPMI. \* P = 0. 0472 for BNN27 versus RPMI group, \*\* P = 0. 0017 for RPMI versus TGF-β+ IL-6+ IL-21+ IL-1β group, \*\*\* P = 0. 0002. Data are expressed as mean ± s. e. m. (J) Percentages of IFN-γ+, IL-17+ and IFN-γ+ IL-17+ cells on human CD4+ T cells. One representative of two independent experiments (K) IFN-γ, IL-17A, TNF-α, IL-4 and IL-10 levels were measured by Cytometric Bead Array assay in the supernatants of human PBMCs from 8 RR-MS patients, cultured for 8hrs in the presence of 100nM BNN27 or 100nM DHEA or RPMI. Data are shown as mean ± SEM (normal distribution) and were compared with Mann-Whitney test analysis using GraphPad Prism 5 software (\* P <0. 05, \*\* P <0. 01, \*\*\* P < 0. 001). BNN27 effects on immune cells are mediated by NGF receptors. (L) Competitive binding assays of a single concentration of [3H]DHEA in the presence of increasing concentrations of BNN27 on membranes isolated from HEK293 cells transfected with the cDNAs of TrkA or p75NTR receptors. Western blot inserts show the efficacy of transfection (Ki represents the mean ±SEΜ of 3 experiments). (M) left panel: CD4+ T cells isolated from day10-EAE mice, were exposed for 20 min to 100 nM BNN27 or 100 nM DHEA or 100 ng/ml NGF, and cell lysates were analyzed by Western blotting, using specific antibodies against the phosphorylated and total forms of TrkA receptor. Right panel: CD4+ T cells were exposed for 20 min to 100 nM BNN27 or 100 nM DHEA or 100 ng/ml NGF, and cell lysates were immunoprecipitated (IP) with p75NTR antibody, followed by immunoblotting (IB) with RIP2 and p75NTR antibodies. (N) Numbers of IL-10+ / 106 CD4+ T cells from cultures of DLN cells from day 10- EAE mice, stimulated ex vivo with MOG35-55, in the presence of 100nM BNN27 or RPMI and anti-p75 or iTrkA or both, for 4 days. \*\* P = 0. 009. Data are expressed as mean ± s. e. m., two independent experiments. Supplementary Materials: Materials and MethodsMice We purchased Balb/c, C57BL/6 (Β6), C57BL/6 Rag1-/-, 2D2 TCR Tg, Il10-/- and Il10gfp (Il10tm1. 1Karp) mice from the Jackson Laboratory. SJL mice were purchased from Charles River Laboratories. Mice were housed at the Animal Facility of the Biomedical Research Foundation of the Academy of Athens. All protocols were in accordance with the US National Institute of Health Statement of Compliance (Assurance) with Standards for Human Care and Use of Laboratory Animals (#A5736-01) and with the European Union Directive 86/609/EEC for animal research. Peptides MOG peptide (amino acids 35–55; MEVGWYRSPFSRVVHLYRNGK) and the peptide of proteolipid protein (PLP; amino acids 139–151; HCLGKWLGHPDKF) were synthesized on a peptide synthesizer (Genemed Synthesis, Inc.) by standard 9-fluorenylmethoxycarbonyl chemistry and were purified by high-performance liquid chromatography. Amino acid sequences were confirmed by amino acid analysis and mass spectrometry. The purity of each peptide was over 95%. Induction of EAE All mice were female, 8-12 weeks old. For SJL/J mice, EAE was induced by immunization with 100μg of PLP139-151. For C57BL/6 mice, EAE was induced by immunization with 200μg of MOG35-55. For 2D2 TCR Tg mice, EAE was induced by immunization with 100μg of MOG35-55. All peptides were dissolved in complete Freund's adjuvant (Sigma-Aldrich) containing 4 mg/ml of heat-killed Mycobacterium tuberculosis H37Ra (Difco Laboratories) as described . On the day of immunization and 48h later, mice were injected intraperitoneally (i. p.) with 300 ng of Bordetella pertussis toxin (List Biological Laboratories, Inc.) in PBS. Mice were examined daily for clinical signs of EAE and were assigned scores as follows: 0, no paralysis; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; and 5, moribund or dead. For each mouse, remission was defined as a decrease in the score of at least one point for at least 2 consecutive days . Administration of BNN27 and DHEA in vivo When C57BL/6 or SJL/J or 2D2 TCR Tg mice had hindlimb paralysis were divided into three groups and then injected intraperitoneally (i. p.) every day with 2mg of DHEA or 2mg of BNN27 in 500μl of PBS. Passive transfer of EAE For adoptive transfer of EAE, female C57BL/6 Il10+/+ or Il10-/- mice, 8–12 weeks old, were immunized subcutaneously (s. c.). with 200μg MOG35-55 in complete Freund's adjuvant containing Mycobacterium tuberculosis H37Ra (4 mg/ml) and treated from the first day of disease onset until disease peak with BNN27 or DHEA or PBS. Draining lymph nodes (DLNs) and splenocytes were collected 17 d after immunization. CD4+ T cells were collected (purified by negative selection, Dynal, Invitrogen) with over 95% purity and washed and 107 cells were transferred i. p. into naive female recipient C57BL/6 Rag1-/- mice. Recipients were induced by immunization with 200μg of MOG35-55 in IFA (incomplete Freund’s adjuvant) (Sigma-Aldrich) on day 0 and given 300 ng of pertussis toxin i. p. on days 0 and 2 after transfer. Mice were examined daily for clinical signs of EAE, as described above (induction of EAE). For adoptive transfers into EAE recipients, T cells (purified by negative selection, Dynal, Invitrogen) or CD4+ T (purified by negative selection, Dynal, Invitrogen) or CD11c+ (purified by positive selection, Miltenyi Biotech) cells, with over 95% purity were collected and washed and 107 cells or 5x106 or 5x105 cells respectively, were transferred i. p. into C57BL/6 EAE mice, with clinical score 1 to 2. In vivo suppression assay Female C57BL/6 mice 8–12 weeks old were immunized s. c. with 200μg MOG35-55 in complete Freund's adjuvant containing Mycobacterium tuberculosis H37Ra (4 mg/ml) and treated from the first day of disease onset until disease peak with BNN27 or DHEA or PBS. DLNs and splenocytes were collected 17 d after immunization. CD4+ T cells were collected (purified by negative selection, Dynal) and washed and 4 x 106 cells were co-transferred i. p. with 4 x 106 CFSE-labeled CD4+ T cells (purified by negative selection, Dynal) from day 10- EAE mice, into age-matched naive female C57BL/6 Rag1-/- recipients. Recipients were immunized s. c. with 200μg MOG35-55 in incomplete Freund's adjuvant (IFA) and were given 300 ng pertussis toxin i. p. on days 0 and 2 after transfer. Mice were examined daily for clinical signs of EAE, as described above. In Fig. 4B, CFSE dilution was assessed by flow cytometry in the DLN cells of recipients, 7 days after the adoptive co-transfer. Anti-IL-10 treatment Mice (n = 5) received 50μg of affinity-purified monoclonal antibody to IL-10 (EBioscience) or functional grade purified rat IgG2b isotype control (EBioscience) i. p. when a clinical score of 1-2 was observed, for 3 times, every second day. Challenge of EAE mice We challenged B6 EAE treated mice with 200μg/mouse MOG35-55 in IFA (1: 1), or with 300ng/ mouse of Bordetella pertussis toxin in PBS, 20 days after EAE induction. In fig. S8, E and F, we challenged B6 EAE treated mice with 0. 01 mg/ mouse Ovalbumin (OVA) (Sigma-Aldrich) in 0. 2 ml CFA. 7 days later mice were sacrificed and the DLN cells were isolated for further analysis. Histology Mice with EAE were sacrificed ~25 days after induction. Spinal cords were fixed in 10% (volume/volume) saline-buffered formalin and were embedded in paraffin. 7-µm paraffin-embedded sections were stained with hematoxylin and eosin (H&E) to evaluate spinal cord infiltration, as described previously . For confocal microscopy experiments, on the day of collection, mice were deeply anesthetized with sodium pentobarbital (Dolethal 0. 7 ml/kg i. p) and were perfused transcardially with heparinized saline solution for about 15 min and then undergone perfusion in 4% PFA, 15% picric acid, 0. 05% glutaraldehyde in PBS 0. 1M, for another 15min. After the perfusion the spinal cords were collected and maintained in the same fixative over night at 4°C. Spinal cords were then washed in 0. 1 M PBS, embedded in 2. 5% agarose and stored at 4°C in 0. 1 M PBS. The samples were sectioned (45μm) in a vibratom and free floating sections were processed for immunostaining. Sections were washed in PBS 0. 1 M then in TBS and incubated for 45 min with 10% horse serum in TBS-T 0. 1%. For MBP staining sections were prefixed for 15 minutes in cold methanol. The normal serum was drained off and the primary antibodies [anti-CD3 1: 50 (eBioscience, 14-0032-66), anti-GFAP 1: 400 (abcam, ab7260), anti-Iba1 1: 400 (abcam, ab576), anti-Olig2 1: 200 (abcam, ab9610), anti-Brdu 1: 200 (abcam, ab6326) and anti-MBP 1: 500 (chemicon, ab980)], diluted in TBS-T 0. 1% with 1% horse serum, were added. Sections were incubated for 4h at RT and overnight at 4°C; they were then washed in TBS-T 0. 1% and the secondary antibodies (Alexa Fluor 488, 546 and 633, 1: 1000 in TBS-T 0. 1%) were added for 6h at RT. Sections washed in TBS-T, TBS and in PBS 0. 1 M counterstained with Hoechst for 5 min at RT then coverslipped with Vectashield (Vector, H-1400) and visualized in a confocal microscope. Preparation of CNS mononuclear cells Spinal cords were flashed out with PBS. Mononuclear cells were isolated by passing the tissue through a cell strainer (70μm; BD Biosciences), followed by a Percoll (Sigma-Aldrich) gradient (70%-30%) centrifugation. Mononuclear cells were removed from the interface, washed and resuspended in culture medium for further analysis. Cell culture, proliferation and cytokine analysis Splenocytes and lymph node cells (5 x 105-106 cells/well) from treated EAE mice were cultured in round-bottomed, 96-well plates in RPMI complete medium (GIBCO) containing 10% (vol/vol) FCS, penicillin-streptomycin, L-glutamine and 2-mercaptoethanol (Invitrogen), with MOG35-55 peptide (25 μg/ml), as previously described . To determine in vivo T-cell function, CD3+ cells were purified by negative selection (Dynal, Invitrogen) from cervical lymph nodes of day 16-MOG-immunized mice and cultured 1: 5 with mitomycin-C-treated syngeneic splenocytes and MOG35-55peptide (25μg/ml). In Fig. 3D and fig. S10C, 2D2 TCR Tg DLN cells were cultured for 24-96 hrs with MOG35-55 in the presence of 100nM BNN27 or 100nM DHEA or RPMI and then CD4+ T cells were analyzed. In fig. S10D, CD4+ T cells were purified (negative selection) from the LNs of naïve BALB/c mice and cultured with anti-CD3 (3μg/ml) and anti-CD28 (2μg/ml) (BD Biosciences) in the presence of 100nM BNN27 or 100nM DHEA or RPMI. For NGFR blocking experiments, the DLN cells from day-10-MOG-immunized mice or from 2D2 TCR Tg mice were cultured for 24-96 hrs with MOG35-55 in the presence of 100nM BNN27 or 100nM DHEA or RPMI, and anti-p75 or a synthetic inhibitor for TrkA or both. For TH17 maintenance experiments, DLN cells from Il10+/+ or Il10-/- EAE mice or sorted IL-10+ and IL-10- CD4+ T cells from the DLNs of Il10gfp EAE mice, were plated in 24-well plates with a-CD3/a-CD28 and cultured in the presence of rIL-6 (0, 1μg/ml) (Peprotech), rTGF-β1 (10ng/ml) (R&D), rIL-23 (20ng/ml) (Peprotech) and MOG35-55, together with 100nM BNN27 or 100nM DHEA or RPMI, for 7 days and then analyzed by flow cytometry or CD4+ T cells were isolated (purified by negative selection, Dynal) for gene expression analysis. DLN cells from EAE-treated mice and challenged with OVA/CFA were re-stimulated ex vivo with 100μg/ml of OVA. Proliferation rate was assessed, as previously described . Cultures were pulsed with [3H]-thymidine (1μCi per well) after 72 h of culture and harvested 16-24h later onto filter paper. The counts per minute (c. p. m.) of incorporated [3H]-thymidine were read using a beta counter. Cytokines were measured in the supernatants of cultured cells using anti-mouse OPTEIA ELISA kits (BD Pharmingen) and DuoSet ELISA Development System (R&D systems). Supernatants were taken at the time of peak production for each cytokine (48 h: Opn, IL-6, TGF-β1; 72h: IFN-γ, TNF-α; 96 h: IL-17Α ; 120h: IL-10). Flow cytometry For intracellular cytokine staining, cells were stimulated for 4-5h in complete medium with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). Surface staining was then done in the presence of Fc-blocking antibodies (Sigma), followed by intracellular staining for cytokines with the Cytofix/Cytoperm kit (BD Biosciences) as directed. Antibodies were from BD Biosciences (CD3, CD4, CD25, IL-10, IL-17Α, IFN-γ, CCR6), EBioscience (Foxp3 staining kit) and Biolegend (TCR Vβ11). In vitro suppression assay Responder CD4+ T cells (CD4+CD62L+CD25-) were sorted (FACSAria™ cell sorter, BD Biosciences) from cervical LNs and splenocytes of 2D2 mice. CD4+ T cells [TCR Vα3. 2+ (Biolegend), CD4+] were sorted from cervical LNs of BNN27- or PBS- treated 2D2 EAE mice. For the suppression assays, 100, 000 responder CD4+ T cells were CFSE-labeled and co-cultured with 105 CD4+ T cells and 2 x 105 mitomycin C-treated syngeneic splenic antigen-presenting cells (APCs) per well in the presence of MOG35–55 (10μg/ml) for 7 days. After 7 days, CFSE dilution was assessed by flow cytometry. CFSE labeling CD4+ T cells were suspended at a density of 5 x 107 cells/ ml in PBS containing 5% (volume/volume) FCS. CFSE (Molecular Probes) diluted in PBS containing 5% (volume/volume) FCS was added to an equal volume of prewarmed cell suspension at a final concentration of 10 mM and the suspension was mixed rapidly. Cells were incubated for 15 min at 37º C, then were incubated for an additional 5 min on ice. At the end of the incubation, cells were washed three times in PBS containing 5% (volume/volume) FCS and were resuspended in the culture medium (described above; cell culture, proliferation and cytokine analysis). Microglia cell culture Primary microglia cells were isolated from 8 weeks old C57BL/6 male mice as previously described . Briefly, mice were deeply anesthetized and perfused with cold PBS with heparin (2U/ml), brains were removed, minced and incubated in an enzymatic solution containing papain (1mg/ml) and dispase II (1. 2U/ml) in room temperature for 30min under continuous rotation. Reaction was stopped by addition of FBS. Cell aggregates were dissociated by DNAse treatment. Isolated cells were plated on poly-L-lysine-coated dishes and cultured in DMEM/F12 supplemented with 10% FBS, 1% P/S and 5ng/ml GM-CSF. Medium was changed for the first time after 4 days of culture and then every two days. After 3 weeks of culture a feeder layer had formed on which microglia cells were growing and migrating to the supernatant, from which microglial cells were collected by centrifugation. Isolated primary microglial cell populations were GFAP-/Iba1+ and > 80% CD68+/ IB4+. BV2 cells were purchased from LGC Standards. BV2 cells were grown in RPMI 10%FBS 1% Pen/Strep at 37°C 5% CO2 in a humidified atmoshpere. Prior to treatments primary microglia cells were starved for 3 days from GM-CSF. Primary microglia cells and BV2 cells were treated with 100nM DHEA or 100nM BNN27 or vehicle (ethanol) in DMEM/F12 supplemented with 1% charcoal-stripped FBS for 24 hrs with or without stimulation with LPS (100ng/ml) and IFN-γ (20ng/ml). Total RNA was isolated, DNase treated, reverse transcribed and analyzed by real-time PCR. Cytokine release from the PBMCs of RR-MS patients Study subjects provided written informed consent for the use of their peripheral blood samples for the purpose of this research. The study was submitted to and approved by the internal review board and scientific advisory committee of Patras University Hospital (ref. 296/23. 9. 08 and 451/17. 10. 08). The Hospital abides by the Helsinki declaration on ethical principles for medical research involving human subjects. Venous blood samples (10 ml) were obtained from 8 RR-MS patients (Table S1) in compliance with institutional review board (IRB) protocols. The concentrations (in pg/ml) of the cytokines IFN-γ, IL-17A, IL-4 and IL-10 were measured in PBMC cultures (106 cells/ ml) in the presence/absence of 107M DHEA or BNN27. Determination of the cytokine concentrations was performed on a BD FACSArray Bioanalyzer using the Cytometric Bead Array (CBA) assay (human Th1/Th2/Th17 Cytokine Kit, BD Biosciences). Human TH17 cell differentiation Peripheral blood was obtained from healthy subjects in compliance with institutional review board (IRB) protocols (as mentioned above) and PBMCs were isolated by Histopaque (Sigma-Aldrich) gradient centrifugation. CD4+ T cells were isolated from PBMCs, by depletion of non-CD4+ T cells (negative selection), using the CD4+ T cell Isolation kit II (Miltenyi Biotec) and were determined to be > 95% CD4+ by flow cytometry. Naive CD4+ T cells were cultured under TH17 polarizing conditions, as previously described , for 6 days at a density of 8 x 104 cells/ well in 48-well plates and stimulated with Dynabeads Human T-Activator CD3/CD28 (one bead per cell) (Dynal), rIL-6 (25ng/ml), rTGF-β1 (5ng/ml), rIL-21 (25ng/ml) and rIL-1β (12, 5ng/ml). DHEA (100nM) or RPMI to control wells, were added daily. After 6 days, cells were collected and washed extensively. Cells were restimulated with Dynabeads CD3/CD28 for 24h (for ELISA; IL-17; EBioscience) or for 5-6h with phorbol 12-myristate 13-acetate (50 ng/ ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug. Surface staining was then done in the presence of Fc-blocking antibodies, followed by intracellular staining for cytokines with the Cytofix/Cytoperm kit. Antibodies were from BD Biosciences (IL-10, IFN-γ) and EBioscience (IL-17). Immunoprecipitation assays and Western Blotting CD4+ cells were incubated with BNN27 100nM or DHEA 100nM or ADIOL 100nM or NGF 100ng/ml for 20min in the presence of serum free, washed twice with ice-cold phosphate-buffered saline (PBS) and suspended in cold lysis buffer (Pierce), supplemented with protease (1 mM PMSF and 1 mg/ml aprotinin) and phosphatase inhibitors (Pierce). Cell lysates were immunoprecipitated (IP) with p75NTR antibody (Promega) overnight at 4oC. Protein G plus agarose beads (Santa Cruz) were then incubated with the lysates for 4h at 4oC with gentle shaking. Beads were collected by centrifugation, washed four times with lysis buffer and resuspended in SDS loading buffer. Proteins were separated by SDS/PAGE followed by immunoblotting with specific antibodies: RICK, Enzo; p75, Promega; GAPDH, Santa Cruz. In the case of phosphorylation of TrkA receptor, the following antibodies were used: phosphoTrkA, Cell Signaling; TrkA, Millipore; GAPDH, Santa Cruz. Proteins were visualized using the ECL Western blotting kit (ECL Amersham Biosciences, UK) and blots were exposed to Kodak X-Omat AR films.[3H]-DHEA Binding Assays HEK293 cells transfected with the cDNAs for TrkA and/or p75NTR (Western Blot inserts show the efficacy of transfection) were cultured, collected by scraping on ice and washed twice with cold Phosphate Buffer Saline (PBS), pH 7. 4. After a centrifugation at 1200 rpm, cells were homogenized by sonication (3 x 30sec, at 4°C) in a 50mM Tris-HCl buffer pH7. 4 (at 4°C) containing freshly added protease inhibitors (1mM PMSF and 1μg/ml aprotinin). Crude membrane fractions were isolated by differential centrifugation at 2, 500g (10 min at 4oC, to remove unbroken cells and nuclei) and 100, 000g (1 h, at 4oC). Membranes were washed once with ice-cold 50 mM Tris– HCl buffer, pH 7. 4, and re-suspended in the same buffer. Membranes were then briefly acidified with 50 mM glycine pH 3 for 3 min on ice to elute membrane adsorbed proteins, washed once, resuspended in PBS (pH 7. 4) with protease inhibitors, at a concentration of 2 mg/ml, and used immediately. A constant concentration of [3H]-DHEA (5nM) was incubated with increasing concentrations of BNN27 (from 10-12 to 10-6 M) or ADIOL (from 10-12 to 10-6 M) in a final volume of 100μl. Membranes were then incubated overnight at 4oC on a rotating plate; then they were collected on GF/B filters and pre-wetted in 0. 5% PEI solution at 4oC. Filters were washed five times with ice-cold PBS, dried and counted in scintillation fluid (SigmaFluor, Sigma) in a scintillation counter (Perkin Elmer, Foster City, CA) with 60% efficiency for Tritium.[3H]-17β-Estradiol Binding Assays HEK293 cells transfected with the cDNAs for ERβ (Western Blot inserts show the efficacy of transfection), were cultured, collected by scraping on ice and washed twice with cold Phosphate Buffer Saline (PBS), pH 7. 4. After a centrifugation at 1200rpm, cells were homogenized by sonication (3 x 30sec, at 4°C) in a 50mM Tris-HCl buffer pH7. 4 (at 4°C) containing freshly added protease inhibitors (1mM PMSF and 1μg/ml aprotinin). Cytoplasmic (supernatant) fractions were obtained by centrifugation at 100, 000g (1 h, at 4oC). For the competitive binding assay, increasing concentrations of BNN27 (from 10-12 to 10-6 M) or ADIOL (from 10-12 to 10-6 M) or Estradiol (from 10-12 to 10-6 M) were incubated with cytosol (~250μg protein) in the presence of 4 nm of [3H] 17β- Estradiol for 18 h at 4oC. Bound and free estradiol were separated using an equal volume of DCC (Dextran-coated charcoal) for 20min at 4oC followed by centrifugation at 3200g for 20 min at 4oC. Bound [3H] estradiol was measured in scintillation fluid (SigmaFluor, Sigma) in a scintillation counter (Perkin Elmer, Foster City, CA) with 60% efficiency for Tritium. RNA isolation and RT-PCR Total RNA was isolated by RNeasy kit (QIAGEN) from cells. One microgram from total RNA was used for cDNA synthesis using Superscript II (Invitrogen), and real-time PCR was performed with SYBRgreen (Invitrogen), detected by Step One Plus (Applied Biosystems). The sequences of RT-PCR primers used for mRNA relative expression analysis in this study were purchased from MWG/Operon and were: Foxp3 FW: CCTCCACTCCACCTAAAG, Foxp3 Rev: TGAAACCAGACAACTAACAG, Il10 FW: AGCTTATCGGAAATGATCC, Il10 Rev: ACTCTTCACCTGCTCCACT, Il10Ra FW: TGTGGCAAAAGGGACTGA, Il10Ra RV: AGTGAGCAGCAGGGAGTAATG, Hprt FW: GTGAAACTGGAAAAGCCAAA, Hprt Rev: GGACGCAGCAACTGACAT, Ccr6 FW: CCCGTGTTGTATGCGTTTAT, Ccr6 Rev: TGCTTTGTGCTCTCGTGTTA, Il17a FW: GCCCTCAGACTACCTCAACC, Il17a Rev: CACACCCACCAGCATCTT, Rorc FW: TGTTTTATGGGGTTTGGGTA, Rorc Rev: AAGAGATTGTGTGCCAGAG. Movies S1-S# (big file)