SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Patients. Consecutive patients with relapsing-remitting MS according to McDonald criteria (Polman et al., 2011) were recruited from the MS clinic at the Raúl Carrea Institute for Neurological Research (FLENI) between September of 2011 and November of 2012. All patients lived in Buenos Aires City (latitude 34.6°S, longitude 58.4°W). Serum and first-morning urine were collected each season between 8 and 9 am during 2011-2012 and stored at -80°C. A second cohort of 26 relapsing-remitting MS patients was recruited between January and February of 2015 and serum and whole blood were collected between 8 and 9 am for $CD4^+$ T cell isolation and melatonin measurement. For each sample, the exact date and time of collection and processing was recorded. Seasons were defined according to the southern hemisphere as follows: Summer (January-March), Fall (April-June), Winter (July-September), Spring (October-December). Study protocol was approved by the Institutional Ethics Committee, and all subjects signed an informed consent form.

Clinical data. Clinical data were retrieved from our MS patient database. The number of relapses occurring from 2007 until 2012 was used to calculate monthly and season exacerbation rate. Exacerbation was defined as development of a new symptom or worsening of a preexisting symptoms confirmed by neurological examination, lasting at least 48 hours, and preceded by stability or improvement lasting at least 30 days.

Melatonin, vitamin D, UVB and infections assessment. Vitamin D levels were quantified at the clinical laboratory of the Raúl Carrea Institute for Neurological Research (FLENI). 6-sulfatoxymelatonin (6-SM), which is the main melatonin metabolite and has an excellent correlation with night-time melatonin levels (Graham et al., 1998), was measured by ELISA as previously described (Graham et al., 1998) (Genway Biotech). For some experiments, serum melatonin was measured using a competitive ELISA kit (Genway Biotech). Official reports of upper respiratory tract infections in Buenos Aires city for the period studied were provided by governmental officials. UV incidence for Buenos Aires location was obtained from NASA satellites trough the Giovanni system (http://disc.sci.gsfc.nasa.gov/giovanni).

Animals and EAE. MTNR1A and ROR-α knockout mice were purchased from Jackson Laboratories. C57BL/6 wild-type were purchased from the Faculty of Veterinary in La Plata University and Jackson Laboratories. NFIL3-deficient mice were provided by Chen Zhu; REV-ERBα deficient mice were provided by Mitch Lazar (University of Pennsylvania, Philadelphia, USA), and C/EBPα knockout mice were provided by Daniel Tenen (Beth Israel Deaconess Medical Center, Boston, USA). EAE was induced as follows: mice were immunized with 100 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) and 500 μg Mycobacterium tuberculosis extract H37Ra (Difco). Mice were also injected intraperitoneally with 200 ng pertussis toxin on days 0 and 2. Melatonin (5mg/kg) or vehicle (0.01% DMSO) was administered daily at 7:00 PM. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School and IBYME.

Isolation of CNS infiltrates. CNS infiltrates were isolated as described (Mascanfroni et al., 2013). Mice were perfused with ice-cold PBS. The brain and spinal cord were removed and incubated in PBS containing collagenase type III (2 mg/ml; Worthington) and DNase (20 units/ml; Sigma-Aldrich). Tissues were then homogenized and loaded on a 30%-37%-70% Percoll gradient for enrichment of CNS infiltrates.

In vitro mouse T-cell differentiation. Naive $CD4^+$ T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^–$) were from the spleen and lymph node of C57BL/6 wild-type, MTNR1A-, NFIL3-, REV-ERBα, C/EBPα-deficient or RORα-deficient mice using magnetic beads ($CD4^+$ T cell isolation kit, Miltenyi Biotec). All experiments were started between 7 and 9 am. Cells were activated with plate-bound anti-CD3 (2 μg/ml; 14-0031-86; eBioscience) and anti-CD28 (2 μg/ml; 16-0281-86; eBioscience). Mouse IL-27 (30 ng/ml; 34-8271; Biolegend) was added for the generation of Tr1 cells. IL-6 (30 ng/ml; 406-ML-025; R&D Systems), TGF-β_1 (15 ng/ml; 130-095-067; Miltenyi Biotec), anti-IL-4 (2.5 μg/ml ;C17.8; Biolegend) and anti-IFN-γ (5 μg/ml;XMG1.2; Biolegend) were added for the generation of Th17 cells. Recombinant mouse IL-23 (30ng/ml; 1887-ML-010; R&D Systems) was added at day 2. For some experiments IL-6 and IL-1β (10ng/ml; 401-ML-025; R&D Systems) or TGF-β_1 and IL-21 (100ng/ml; 594-ML-010; R&D

Systems) were used in instead for Th17 cell differentiation. IL-12 (30ng/ml; 419-ML-010; R&D Systems) and anti-IL-4 (2.5 μg/ml ;C17.8; Biolegend) were used for the generation of Th1 cells. IL-4 (30ng/ml; 404-ML-010; R&D Systems) and anti-IFN-γ (5 μg/ml;XMG1.2; Biolegend) was added for the generation of Th2 cells. TGF-β_1 (15 ng/ml; 130-095-067; Miltenyi Biotec) was used for the generation of $Foxp3^+$ Tregs. Melatonin (Gador, Argentina), Agomelatin, and CGP-52608 (Sigma-Aldrich) were added at the start of the cultures and at day 2, at a final concentration of 2-20ng/ml.

In vitro human T cell differentiation. For Th17 differentiation, naive $CD45RA^+$ $CD4^+$ T cells were isolated from PBMCs with magnetic beads (Naive Human $CD4^+$ T Cell Isolation Kit II, Miltenyi Biotec) and seeded at a density of 5×10^5 cells/ml in 24-well plates coated with anti-CD3 and (2 μg/ml) and soluble anti-CD28 and cultured in the presence of the following cytokines IL-1β (25 ng/ml), IL-6 (50 ng/ml), and TGF-β1 (2 ng/ml) and neutralizing antibodies to IFN-g (10mg/ml) and IL-4 (10 microgram/ml). Alternatively, Th17 cells were differentiated by using IL-1β (25 ng/ml), IL-6 (50 ng/ml), and IL-23 (50 ng/ml) and neutralizing antibodies to IFN-g (10mg/ml) and IL-4 (10 microgram/ml). For Th1 differentiation naïve CD4+T cells are cultured in the presence of IL-12 (20ng/ml) and anti-IL-4 (10 μg /ml).

Measurement of cytokines. Secreted cytokines were measured in tissue culture supernatants after 72-96hs by enzyme-linked immunosorbent assay as previously described (Farez et al., 2009).

Quantitative RT-PCR. Primers-probe mixtures for mouse experiments were as follows (from Applied Biosystems; identifiers in parentheses): rorc (Mm01261022_m1), il23r (Mm00519942_m1), il10 (Mm0043614_m1), il17 (Mm00439619_m1), il21 (Mm00517640_m1), rora (Mm01173766_m1), rorc (Mm00441144_g1), foxp3 (Mm00475156_m1), tbx21 (Mm00450960_m1), gata3 (Mm00484683_m1), nr1d1 (Mm00520708_m1), nfil3 (Mm00600292_s1) and gapdh (Mm99999915_g1). Primers-probe mixtures for human experiments were as follows (from Applied Biosystems; identifiers in parentheses): *RORC* (Hs01076122_m1), *IL17A* (Hs00174383_m1), *IL17F* (Hs00369400_m1), *IL10* (Hs00961622_m1), *IFNG* (Hs00989291_m1) and 18s (Hs03003631_g1).

Chromatin immunoprecipitation. DNA-protein complexes in cells were crosslinked with 4% paraformaldehyde and lysed with 0.35 ml lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1) containing 1×protease inhibitor 'cocktail' (Roche Molecular Biochemicals). Chromatin was sheared by sonication and supernatants collected after centrifugation were diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8.1). 5 μg antibody was prebound for a minimum of 6 hs to protein A and protein G Dynal magnetic beads (Invitrogen) and samples were washed three times with ice-cold PBS containing 5% BSA, and then were added to the diluted chromatin, followed by immunoprecipitation overnight. The magnetic bead–chromatin complexes were then washed three times in radioimmunoprecipitation buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40 and 0.5 M LiCl), followed by two washes with Tris- EDTA buffer. Immunoprecipitated chromatin was then extracted with a solution of 1%SDS and 0.1 MNaHCO3 and was heated at 65°C for at least 6h for reversal of the paraformaldehyde cross-linking. DNA fragments were purified with a QIAquick DNA purification Kit (Qiagen) and were analyzed by SYBR Green real-time PCR (Takara Bio).

Signaling arrays. Cells activated under polarizing conditions were treated with vehicle (DMSO 0.001%), melatonin (2-10ng/mL), agomelatine (2-10ng/mL) and CGP-52608 (2-10ng/mL) during 72-96hs and lysed. Lysates were transferred to 384-well polypropylene plates and were spotted onto Super Epoxi slides (Telechem) with a robotic microarrayer (Genetix) fitted with solid spotting pins. Slides were then probed, processed and analyzed as described (Farez et al., 2009).

Proliferation assays. Splenic cells were obtained from vehicle or melatonin treated WT mice 10 days after immunization with $MOG_{35–55}$ and were re-stimulated *in vitro* for 3 days in the presence of $MOG_{35–55}$. The cells were pulsed with [3H]thymidine (1 μCi/well) for the final 24 h. The frequency of T cells producing IL-17 (eBioscience), IFN-γ (BioLegend) or IL-10 (BD Pharmingen) and Foxp3+ T cells (eBioscience) was assessed by flow cytometry. For CFSE-based proliferation assay, $CD4^+$ T cells were labeled with 1 μM CFSE (carboxyfluorescein diacetate succinimidyl ester; Molecular Probes). Data were acquired on an LSR III (BD Biosciences) or MacsQuants (Miltenyi) and analyzed with FlowJo software (TreeStar).

Plasmids. The IL-10 promoter reporter and C-Maf and AhR vectors were previously described (Apetoh et al., 2010), vector expressing ROR-α were purchased from PlasmID at Harvard Medical School. Vectors coding for C/EBPα (44627) and Bmal reporter (46824) were purchased from Addgene. The retrovirus used for *nfil3* overexpression in T cells was graciously provided by Laura Hooper (UT Southwestern, TX, USA). The retrovirus used for *nr1d1* overexpression in T cells was graciously provided by Bart Staels (Institut Pasteur, Lille, France, USA). The *nr1d1* promoter reporter was graciously provide by Vincent Laudent (Ecole Normale Supérieure, Lyon, France).

Transfection and luciferase assays. HEK293 cells were grown in DMEM supplemented with 10% FBS and were transfected with FuGENE HD transfection reagent and 2 μg of each plasmids according the manufacturer's instructions (Roche). Firefly and renilla luciferase activity was analyzed 48 h after transfection and 24 h after treatment with a Dual Luciferase Assay kit (Promega).

Retroviral transduction. Retroviral expression constructs were transfected into human embryonic kidney HEK293T cells along with eco and gag-pol viral envelope constructs. Viral supernatants were collected at 72 h after transfection. Lentiviral transduction was performed by spinoculation at 1200*g* for 1 hr at 32°C in the presence of polybrene (8 μg/ml; Sigma).

T-cell transfer and immunization. Sorted splenic $CD4^+$ T cells from C57BL/6, MTNR1A-, REV-ERBα and NFIL3-deficient mice were transferred i.p. ($10x10^6$ cells per mouse) into RAG-1 deficient mice. Ten days after transfer, mice were checked for reconstitution of $CD4^+$ T cells and immunized with MOG_{35-55} in CFA. Twenty days after immunization, T cells were isolated and stained for cytokines.

SUPPLEMENTAL TABLES

Table S1. Clinical features of EAE, Related to Fig. 2a

Group	Incidence (%)	Mortality	Mean onset day (mean±sd)	Mean maximum score (mean±sd)
Vehicle	17/20 (87.5%)	0/20	12.8±3.2	3.05±1.4
Melatonin	17/24 (65%)*	0/24	11.8±3.0	2.06±0.8**

* P=0.05
** P<0.05

Table S2. Baseline and clinical characteristics of the MS cohort used for expression studies. Related to "Melatonin affects human T-cell differentiation" section.

	All participants (n=26)
Age (years, mean ± SD)	38 ± 9.24
F:M (n)	13:13
Disease duration (years, median, range)	5 (1-14)
EDSS (median, range)	1 (0-4)
Treatment (n)	
Interferon	6
Glatiramer Acetate	4
Fingolimod	11
Other	5

Table S3. Correlation between melatonin levels and IL10 and IL17F in $CD4^+$ cells isolated from MS patients. Related to "Melatonin affects human T-cell differentiation" section.

Variable	Coefficient	Standard Error	95% CI	P value
IL10	0.009	0.00053	0.007 - 0.011	0.003
IL17	-3.92	0.89	-6.4 - -1.4	0.012
RORC	0.001	0.00111	-0.004 – 0.003	0.387
NR1D1	0.00000951	0.0000625	-0.0002 – 0.0001	0.882
NFIL3	0.00013	0.00008	-0.00006 – 0.0003	0.163

SUPPLEMENTAL REFERENCES

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