Published in final edited form as:

Nat Immunol. 2015 December ; 16(12): 1228–1234. doi:10.1038/ni.3321.

The orphan nuclear receptor Nr4a1 couples sympathetic and inflammatory cues in CNS-recruited macrophages to limit neuroinflammation

Iftach Shaked^{2,1}, Richard N. Hanna^{2,1}, Helena Shaked², Grzegorz Chodaczek², Heba N. Nowyhed², George Tweet², Robert Tacke², Alp Bugra Basat², Zbigniew Mikulski², Susan Togher², Jacqueline Miller², Amy Blatchley², Shahram Salek-Ardakani³, Martin Darvas⁴, Minna U. Kaikkonen⁵, Graham Thomas², Sonia Lai-Wing-Sun⁶, Ayman Rezk⁶, Amit Bar-Or⁶, Christopher K. Glass⁷, Hozefa Bandukwala², and Catherine C. Hedrick²

²La Jolla Institute for Allergy and Immunology ³University of Florida, College of Medicine

⁴University of Washington ⁵University of Eastern Finland ⁶Montreal Neurological Institute, McGill University ⁷University of California, San Diego

Abstract

Molecular mechanisms linking the sympathetic stress response and inflammation remain enigmatic. Here we demonstrate that the transcription factor Nr4a1 regulates production of norepinephrine (NE) in macrophages, thereby limiting experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Lack of Nr4a1 in myeloid cells led to enhanced NE production, accelerated leukocyte infiltration to the central nervous system (CNS) and disease exacerbation *in vivo*. In contrast, myeloid-specific deletion of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, protected against EAE. Further, we found that Nr4a1 repressed autocrine NE production in macrophages by recruiting the corepressor CoREST to the *Th* promoter. Our data reveal a new role for macrophages in neuroinflammation and identify Nr4a1 as a key regulator of macrophage catecholamine production.

Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS) that has long been associated with stress and the sympathoadrenergic response^{1,2}. The sympathetic hormone norepinephrine (NE), a major mediator of response to physiological and psychological stressors, was shown to have a role in the pathology of

Correspondence should be addressed to I.S. (Iftach@lji.org) or C.C.H. (hedrick@lji.org).

¹Equal contribution

Author contributions:

IS, RNH, and HS designed, performed and analyzed the experiments;

GC, HNN, and RT designed and performed the experiments;

GT, RT, ABB, ZM, ST, JM, AB, MUK, SLWS, and AR performed the experiments;

SSA, MD, GTh, ABO, CKG, HB, and CCH designed the experiments;

IS, RNH, HS, and CCH wrote the manuscript.

The authors declare no competing interests.

experimental autoimmune encephalitis (EAE), the rodent model of MS^{3–6}. However the molecular mechanisms linking stress signaling to neuroinflammation remain uncertain.

The Nr4a orphan nuclear receptors, Nr4a1 (Nur77), Nr4a2 (Nurr1) and Nr4a3 (Nor1), are early-immediate response genes rapidly induced by a variety of physiological stimuli⁷. Nr4a1, like other Nr4a members, is involved in early sympathetic stress response in the neuroendocrine system^{8–11}. At the same time, Nr4a1 plays an important role in leukocytes, where it is a central regulator of innate and adaptive immune responses. As such, Nr4a1 is involved in activation and differentiation of macrophages in atherosclerosis^{12,13}, and also controls differentiation and survival of nonclassical Ly6C[–] patrolling monocytes^{14,15}. Furthermore, Nr4a1 is rapidly induced in T cells following T cell antigen receptor (TCR) activation and reflects the strength of TCR signaling¹⁶. Thus Nr4a1 is involved both in immunity and in the stress response and it may therefore represent a key junction in the crosstalk between sympathetic and immune systems, particularly in the context of neuroinflammation.

To test this hypothesis, we utilized an established model of EAE and found that mice lacking Nr4a1 developed accelerated and exacerbated disease, which was accompanied by high concentrations of NE and interleukin 6 (IL-6) and early auto-aggressive T cell infiltration to the CNS. Mechanistically, we discovered that Nr4a1 inhibited macrophage expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for NE production¹⁷. We found that myeloid-specific TH deletion protected mice from the disease. Our data demonstrate a major role for macrophage NE production in neuroinflammation, and identify an important mechanism of NE regulation by Nr4a1.

Results

Nr4a1 expression in myeloid cells limits EAE severity

To determine if Nr4a1 plays a role in CNS autoimmunity, we analyzed Nr4a1 expression in mice during EAE. We utilized the passive EAE model (Supplementary Fig. 1a), which involves adoptive transfer of autoreactive CD4⁺ T lymphocytes from myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgenic mice (2D2)¹⁸ that are differentiated *in vitro* to T helper type I (T_H1) cells (Supplementary Fig. 1b). To address Nr4a1 expression in EAE at the cellular level, we induced the disease in mice expressing green fluorescent protein (GFP) under control of the *Nr4a1* promoter¹⁶. Using intravital microscopy, we observed significant induction of Nr4a1-GFP expression in the spinal cord upon EAE onset (Fig. 1a). To determine the identity of the Nr4a1-GFP-expressing cells, we used flow cytometry (Fig. 1b). We identified microglia as CD45^{lo}CD11b⁺ (Ref. 19) whereas CD45^{high}CD11b⁺ identified infiltrating myeloid cells. CD45^{hi}CD11b⁺ infiltrating myeloid populations were further gated to distinguish between granulocytes (Ly6G⁺MHCII[–]), monocytes (Ly6G[–]MHCII[–]) and macrophages (Ly6G[–]MHCII⁺). Macrophages identified by flow cytometry in the CNS were also positive for core tissue macrophage markers CD64 and the receptor MerTK²⁰, while monocytes characteristically expressed some CD64, but not MerTK²¹ (Supplementary Fig. 1c). Nr4a1-GFP was highly expressed in infiltrating macrophages, and to a lesser extent in infiltrating monocytes, as well as in resident microglia

(Fig. 1c). Nr4a1 expression was relatively low in granulocytes and other infiltrating cells (which are likely to be non-2D2 lymphocytes) (Fig. 1c).

To address the importance of Nr4a1 expression in CNS autoimmunity, we induced EAE in *Nr4a1*^{-/-} mice²², which resulted in much earlier onset and exacerbated disease development (Fig. 2a and Supplementary Fig. 2a,b), as well as accelerated body mass loss (Supplementary Fig. 2c), compared to wild-type (WT) mice. *Nr4a1*^{-/-} mice were also more susceptible than were control mice to EAE induced by active immunization with MOG_(35–55) peptide (Supplementary Fig. 2d).

As we observed in Nr4a1-GFP mice, Nr4a1 was highly expressed in myeloid cells in the CNS upon EAE onset. Therefore, to address the role of Nr4a1 in myeloid cells, we utilized mice with a specific Nr4a1 deletion - *Nr4a1*^{fl/fl} mice carrying one of two well characterized myeloid-specific transgenes, *LysM*-Cre (*Nr4a1*^{LysM}) or *Csf1r*-Cre (*Nr4a1*^{Csf1r}). We first analyzed Nr4a1 mRNA expression and deletion efficacy in the myeloid cells in these strains. In wild-type naïve mice, Nr4a1 mRNA was most highly expressed in monocytes and macrophages, with lower expression in granulocytes and microglia. *LysM*-Cre expression significantly reduced Nr4a1 mRNA expression in macrophages, monocytes and granulocytes, but not in microglia, while *Csf1r*-Cre expression effectively reduced Nr4a1 mRNA expression in all myeloid populations (Fig. 2b). Mice with myeloid-specific Nr4a1 deletion using both *LysM*-Cre and *Csf1r*-Cre drivers developed substantially exacerbated EAE compared to control *Nr4a1*^{fl/fl} littermates (Fig. 2c,d and Supplementary Fig. 2e). To test a potential role of Nr4a1 in T cells in EAE onset, we transferred wild-type mice with *Nr4a1*^{-/-} 2D2 autoimmune T cells. In contrast to the myeloid deletion of *Nr4a1*, T cell-specific Nr4a1 loss had no significant effect on disease outcome (Supplementary Fig. 2f). Collectively these data suggest that Nr4a1 expression in infiltrating monocytes and derived macrophages may play a protective role in EAE.

To further characterize the phenotype of *Nr4a1*^{-/-} mice, we determined the identity of inflammatory cells in the CNS by flow cytometry. To track myeloid cells in vivo, we utilized heterozygous CX3CR1-GFP reporter mice on a B6 background or on a *Nr4a1*^{-/-} background, in which macrophages and microglia express GFP^{23–26}. In this setting, microglia were characteristically CD45^{lo}CX3CR1-GFP^{hi}, while infiltrating monocytes/macrophages were CD45^{hi}CX3CR1-GFP^{lo}CD11b^{hi} (Fig. 2e). To track CNS-infiltrating 2D2 T cells, we used 2D2 cells expressing DsRed²⁷ for injection into recipient mice. The majority (68%) of the inflammatory CNS infiltrate in wild-type mice was accounted for by 2D2 cells, while 24% were infiltrating CD11b^{hi} myeloid cells (Fig. 2e). In *Nr4a1*^{-/-} mice, infiltration of total leukocytes (all CD45⁺) and specifically 2D2 T cells was about 6-fold higher than in wild-type mice. Among the infiltrating myeloid cells, macrophages represented the primary cell population (~80%), and their numbers were significantly increased in *Nr4a1*^{-/-} compared to wild-type mice (Fig. 2e).

Infiltrating monocyte numbers were also increased in *Nr4a1*^{-/-} mice compared to wild-type mice. Most of the infiltrating monocytes in both strains consisted of inflammatory Ly6C⁺ monocytes (Supplementary Fig. 3a). Non-classical Ly6C⁻ monocytes represented only a minor fraction in wild-type mice and were almost completely absent in *Nr4a1*^{-/-} mice

(Supplementary Fig. 3a), which is in line with the previously described role of Nr4a1 in Ly6C⁺ monocyte differentiation¹⁵. In EAE, studies have shown that Ly6C⁺ inflammatory monocytes are recruited to the CNS and give rise to macrophages, and that infiltrating Ly6C⁺ monocyte and derived macrophage populations are critical for EAE development^{23,28–30}. Using Nr4a1-GFP mice, we found that Nr4a1 was most highly expressed in CNS-isolated Ly6C⁺ monocytes, but also highly expressed in Ly6C⁺ monocytes during disease (Supplementary Fig. 3b), suggesting an important role of Nr4a1 in regulating these cell types in EAE.

Using intravital microscopy, we confirmed earlier immune cell infiltration and exacerbated CNS damage in *Nr4a1*^{−/−} mice. At 4 days post T cell transfer, few pioneering DsRed⁺ 2D2 T cells were found infiltrating the nervous tissue of the thoracic spinal cord segment in both wild-type and *Nr4a1*^{−/−} mice (Fig. 2f and Supplementary Fig. 4a). In contrast, at day 7, we found greater infiltration of DsRed⁺ 2D2 T cells into *Nr4a1*^{−/−} spinal cords, whereas in wild-type mice, 2D2 infiltration remained confined to an area near the posterior spinal vein (PSV) (Fig. 2f and Supplementary Fig. 4b: see also Supplementary Movies 1,2). Vascular permeability in brain was similar between wild-type and *Nr4a1*^{−/−} mice (Supplementary Fig. 4c), indicating that the increase in T cell infiltration in the *Nr4a1*^{−/−} mice was not caused by impaired blood brain barrier function.

Microglial activation is associated with morphological changes of the cell body and loss of dendritic “ramified” projections seen in the homeostatic state³¹. Consistently, at day 7 after T cell transfer, ramified microglia were severely reduced and rounded microglia were increased in *Nr4a1*^{−/−} mice compared to wild-type in the spinal cord (Fig. 2f and Supplementary Fig. 5a) and brain (Supplementary Fig. 6). Flow cytometry analysis of CD44 and MHCII, markers of activated microglia, further confirmed early microglia activation in *Nr4a1*^{−/−} mice (Supplementary Fig. 5b,c). Interestingly, we also found that at day 7, *Nr4a1*^{−/−} mice had higher blood concentrations of NE, IL-6 and the IL-6 associated chemokine CXCL1 (Fig. 2g) as compared to wild-type mice. Taken together, these data show that *Nr4a1*^{−/−} mice develop early and exacerbated EAE, which involves higher blood induction of NE and IL-6, accelerated CNS recruitment of autoreactive T cells, and early activation of microglia.

Due to higher NE concentrations in *Nr4a1*^{−/−} mice upon EAE induction, we hypothesized that *Nr4a1*^{−/−} mice expressed higher amounts of TH in their spinal cord early in EAE development, as compared to wild-type mice. To exclude changes resulting from the more advanced disease stage in *Nr4a1*^{−/−} mice, we compared wild-type and *Nr4a1*^{−/−} mice with the same disease score. As we expected, in early EAE stages and even before disease induction, *Nr4a1*^{−/−} mice showed increased abundance of mRNAs encoding TH, IL-6 and the IL-6-induced chemokines Ccl5 and Cxcl1 (Fig. 2h). This finding is consistent with a previous report showing higher TH expression in the CNS of *Nr4a1*^{−/−} mice³². Our data therefore suggest that Nr4a1 regulates production of NE and IL-6, thereby limiting EAE susceptibility.

NE-producing myeloid cells have an essential role in EAE

To test whether excessive NE production contributed to EAE severity in *Nr4a1*^{-/-} mice, we applied adrenergic inhibitors blocking autocrine and paracrine NE signaling. Blockade of $\alpha 1$ (Fig. 3a), but not $\beta 1$ or $\beta 2$ (Supplementary Fig. 7a,b) adrenergic receptors significantly reduced EAE progression in *Nr4a1*^{-/-} mice. Catecholamine depletion with the noradrenergic neurotoxin 6-hydroxydopamine (6-OHDA) also inhibited EAE progression (Fig. 3b) in both wild-type and *Nr4a1*^{-/-} mice, highlighting the critical role of catecholamines in the development of EAE.

To identify the cellular source of NE in EAE, we analyzed cells isolated from CNS of EAE mice for the expression of TH using flow cytometry. Interestingly, we found that TH expression was increased during the course of EAE in macrophages and to a lesser extent in monocytes and microglia, but not in granulocytes (Fig. 3c). As macrophages represent a principal component of the CNS infiltrate, these results imply that TH⁺ recruited macrophages may be a major driving force behind EAE development.

To test this possibility, we utilized the dopamine-deficient floxed stop (DDfs) mice³³, in which TH expression is significantly reduced (Supplementary Fig. 7c). Wild-type mice transplanted with DDfs bone marrow were protected from EAE development (Fig. 3d). To further substantiate the role of myeloid TH in driving EAE, we utilized mice with selective deletion of the *Th* gene in LysM⁺ myeloid cells. These mice had reduced TH expression in the CNS-infiltrating myeloid cells compared to wild-type mice, but not in microglia or other immune cells (Supplementary Fig. 7d), and also showed lower blood NE concentrations (Supplementary Fig. 7e). These mice with myeloid-specific deletion of TH were protected from EAE disease progression (Fig. 3e). That TH expression in granulocytes did not change with disease progression, and that *LysM*-driven TH deletion, as well as transfer of DDfs bone marrow, reduced EAE severity while retaining microglial TH expression, further suggested that TH-expressing infiltrating monocytes and derived macrophages are the important cells for EAE development.

Mechanistically, we hypothesized that NE secreted from macrophages during EAE may activate them in an autocrine manner, thereby amplifying the neuro-inflammatory cascade, which leads to T cell recruitment to the CNS. To test this hypothesis, we utilized bone-marrow-derived macrophages (BMMs) as a model for monocyte-derived macrophages since these cells have been shown to both produce NE³⁴ and express adrenergic receptors³⁵. BMMs treated with NE upregulated *Il6* and this upregulation was abolished by the $\alpha 1$ adrenergic blocker and to a lesser extent by the $\beta 1$ adrenergic blocker (Fig. 3f), suggesting that macrophages can sense NE and produce IL-6 in response. *Il6* expression was also reduced by the TH inhibitor α -methyl-p-tyrosine (AMPT) in IFN- γ -treated macrophages (Fig. 3f). Upon IFN- γ stimulation, BMMs from DDfs mice showed much lower expression of *Il6* and genes encoding IL-6-driven chemokines than did wild-type BMMs (Fig. 3g), further confirming a role for autocrine catecholamine production in macrophage neuroinflammatory signaling. Collectively, these results suggest that in early stages of EAE, monocytes and macrophages induce TH and secrete NE, which results in production of IL-6 and IL-6-associated chemokines, thus driving leukocyte recruitment to CNS.

Nr4a1 is a negative regulator of TH in macrophages

Given the essential role of macrophage TH in EAE and our observation that EAE susceptibility of *Nr4a1*^{-/-} mice was accompanied by higher TH expression, we asked whether Nr4a1 could play a relevant role in TH regulation in macrophages. Similar to high Nr4a1 expression *in vivo* in macrophages during neuroinflammation, expression of both *Nr4a1* mRNA and Nr4a1-GFP was increased in BMM stimulated with either IFN- γ or NE (Supplementary Fig. 8a–c), suggesting a role for Nr4a1 as a sensor of both sympathetic and inflammatory stress in macrophages. We therefore asked if Nr4a1 could directly regulate expression of TH in macrophages as a feedback regulation mechanism. Compared to wild-type BMMs, BMMs from *Nr4a1*^{-/-} mice showed markedly increased TH mRNA (Fig. 4a) and protein (Fig. 4b) at baseline and when treated with IFN- γ . *Nr4a1*^{-/-} macrophages also secreted significantly more NE (Supplementary Fig. 8d), which was inhibited by the TH inhibitor AMPT, or by 6-OHDA, (Supplementary Fig. 8d). Therefore, lack of Nr4a1 increases TH expression and NE production in macrophages.

To unravel the molecular mechanism of TH regulation by Nr4a1, we utilized the mouse macrophage cell line Raw 264.7 (RAW), as these cells were also shown to both produce and sense NE³⁶. *Nr4a1* overexpression in RAW cells downregulated *Th* mRNA and conversely, *Nr4a1* knockdown upregulated *Th* mRNA (Fig. 4c). Moreover, loss of Nr4a1 led to increased activity of a 4.5 kb fragment of the murine *Th* promoter using a promoter-reporter assay both in BMM and in RAW cells (Supplementary Fig. 8e) suggesting that Nr4a1 represses *Th* gene transcription. Collectively, these data identify Nr4a1 as an important negative regulator of TH expression in macrophages.

Another Nr4a member, Nr4a2, was shown to bind to the promoter of the *Th* gene in developing neurons^{37,38} and downregulate *Th* expression by recruiting the co-repressor CoREST³⁹. CoREST is known to suppress transcription of neuron-specific genes in a complex with histone deacetylase (HDAC)^{40,41}. Recently, Nr4a1 was also shown to suppress transcription by recruiting the CoREST complex^{42,43}. Because Nr4a1 and Nr4a2 share the same minimal DNA binding sequence⁷, we hypothesized that Nr4a1 could also downregulate *Th* transcription by recruiting the CoREST complex.

CoREST knockdown using siRNA significantly increased *Th* mRNA expression in RAW cells (Fig. 4c). Further, we were able to detect direct interaction between Nr4a1 and CoREST in RAW cells by co-immunoprecipitation (Fig. 4d) and also detected Nr4a1-dependent binding of CoREST to the previously described Nr4a binding sites (NBS) in the *Th* promoter³⁷ using chromatin immunoprecipitation (ChIP) (Fig. 4e, left). ChIP analysis also showed increased abundance of acetylated histone H3 in the *Th* promoter following *Nr4a1* knockdown and, to a higher extent, following CoREST knockdown (Fig. 4e, right), confirming the notion that CoREST regulates *Th* transcription by recruiting HDAC. Taken together, these results suggest that Nr4a1 directly suppresses transcription of the *Th* gene in macrophages by recruiting the CoREST complex to the *Th* promoter.

TH is increased in the monocytes of MS patients

Previous studies have shown that CNS-infiltrating monocytes and monocyte-derived macrophages are crucial for EAE pathogenesis^{23,28–30}. Our data indicate that monocytes and macrophages isolated from CNS during EAE express high amounts of TH and that myeloid TH expression is important for EAE development. TH expression was reported in human and mouse monocyte-derived macrophages^{34,44,45}, but its importance in neuroinflammation was not studied. To address the relevance of TH-expressing monocytes/macrophages to human MS, we analyzed peripheral blood mononuclear cells (PBMCs) obtained from MS patients and age- and sex-matched healthy volunteers (Fig. 5a). We were able to detect TH in both monocyte and lymphocyte cell populations in all the samples, however TH expression was highest in monocytes compared to all other circulating cells (Fig. 5b). Supporting our mouse data, MS patients exhibited higher TH expression in CD14⁺ monocytes compared to healthy controls (Fig. 5c). As classical monocytes differentiate into macrophages once they infiltrate the CNS during EAE onset^{23,28,30}, these data suggest that TH-expressing monocytes and derived macrophages may also play a role in human MS.

Discussion

The main objective of this study was to identify functional and regulatory mechanisms linking stress signaling and neuroinflammation. Adrenergic signaling has long been associated with EAE, however a role for NE-producing macrophages was not suggested. A series of publications in 1980s and early 1990s^{4,5,46,47} showed that prazosin, an α -adrenergic blocker, had a protective effect in both active and passive EAE models in Lewis rats, which is in agreement with our work. The authors suggested that prazosin might exert its effect by stabilizing the BBB; however since vascular leakage correlated with the EAE score in both control and treated animals^{46,47}, the decreased BBB leakage might be secondary to the overall disease suppression by prazosin. We found no gross vascular permeability changes in *Nr4a1*^{-/-} mice, thus the *Nr4a1* protective role in EAE cannot be attributed to changes in vascular permeability. Nevertheless adrenergic signaling effects on vascular endothelial cells may indeed play a role in EAE, such as by inducing IL-6 production and facilitating leukocyte recruitment to CNS³. Moreover, our findings imply that monocytes and macrophages might be a major source of NE for triggering this endothelial activation cascade, in addition to the autocrine signaling we have described.

The main myeloid populations isolated from CNS during EAE include infiltrating monocytes, monocyte-derived macrophages, microglia and granulocytes. Previous studies have shown that recruited monocyte-derived macrophages are critical for EAE development^{23,28–30}. Macrophages have been shown to express TH and produce catecholamines in mice and humans^{34,36,44,45,48}. In line with these reports, we show a disease-promoting role for TH in recruited macrophages and identify a novel mechanism of neuroprotection by *Nr4a1*-mediated TH repression. We observe high expression of *Nr4a1* and TH in both infiltrating monocytes and derived macrophages, and to a lesser extent in microglia. There is relatively low expression of both *Nr4a1* and TH in granulocytes, suggesting against *Nr4a1*-mediated regulation of TH activity in granulocytes. EAE modulation in mice with *Nr4a1* and TH deletions driven by the *LysM*-Cre transgene, which

drives deletion in macrophages and monocytes but not in microglia, indicates that microglia might be less important for the Nr4a1-mediated neuroprotective mechanism described here. Importantly, circulating CD14⁺ monocytes isolated from MS patients have increased TH expression, further suggesting the disease-promoting role of TH in monocytes and derived macrophages. Nevertheless we cannot exclude that the lack of patrolling monocytes in *Nr4a1*^{-/-} mice¹⁵ may contribute to their EAE susceptibility; although this cell type constitutes a relatively small part of the myeloid infiltrate in the CNS, these cells may have a protective role by maintaining vasculature homeostasis.

Both pro- and anti-inflammatory roles for Nr4a1 have been described in various functional settings, including its modulation of the NF- κ B pathway^{49,50}. Another Nr4a member, Nr4a2 (Nurr1), expressed in microglia and astrocytes, was shown to mediate neuroprotection by recruiting the CoREST transrepressor to promoters of NF- κ B target genes⁵¹. However, our data demonstrate an alternate neuroprotective function for Nr4a1 in EAE, which entails suppression of NE production in macrophages through recruitment of CoREST/HDAC1 complex to the promoter of TH. Interestingly, reduced Nr4a1 expression was found in PBMC of MS patients prior to disease onset^{52,53}, supporting a protective role for Nr4a1 in MS. Future studies will further examine the role of Nr4a1 mediated TH regulation in human monocyte subsets and derived macrophages in MS.

Recent studies describe the necessity of neuroimmune communication in controlling inflammation^{3,54}, yet the molecular mechanisms that regulate it are still unclear. Our work suggests a pivotal role for TH-producing myeloid cells and for neuroimmune regulators like Nr4a1 in controlling CNS inflammation. This work also opens new avenues for potential MS therapies via manipulating the amounts or activity of Nr4a1 to regulate myeloid adrenergic responses.

Methods

Mice—C57BL/6J wild-type mice (JAX Stock #000664), *Nr4a1*^{-/-} mice²² (Jax #006187, backcrossed in our lab for 15 generations) and 2D2 transgenic mice expressing a T-cell receptor (TCR) specific for the myelin oligodendrocyte (MOG_{35–55}) peptide (Jax #006912)¹⁸ on a congenic C57BL/6J background were obtained from The Jackson Laboratory. *Nr4a1*^{fl/fl} mice were provided by Daniel Metzger and Hiroshi Ichinose⁶². The Nr4a1-GFP reporter mice have been previously described¹⁶ and are available from The Jackson Laboratory (016617). DDfs mice (contributed by Martin Darvas) have a nonfunctional TH gene because of insertion of a NeoR gene in the first intron of the *Th* gene³³. They were maintained by giving L-Dopa to the dam until their weaning and then bone marrow was prepared. *Th*^{LysM} mice were generated by crossing *LysM*-Cre⁺ mice with *Th*^{fl/fl} mice⁵⁵. Mice were fed a standard rodent chow diet and were housed in microisolator cages in a pathogen-free facility. Mice were euthanized by CO₂ inhalation. All experiments followed guidelines of the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, and approval for use of rodents was obtained from the La Jolla Institute for Allergy and Immunology according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

EAE induction—EAE induction was performed as previously described⁵⁶. Briefly, CD4⁺T cells isolated from 2D2 TCR-transgenic mice¹⁸ were differentiated under T_H1 conditions: with plate-bound anti-CD3, anti-CD28 in the presence of IL-12 (20 U/ ml). On day 5 after initial activation, the cells were harvested and restimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 (20 U/ ml) and IL-18 (25 ng/mL) overnight. Subsequently, the cells were harvested, washed with PBS, and $0.5\text{--}2 \times 10^6$ cells were transferred i.p. into lightly irradiated (400 Rads) 8- to 12-wk-old recipient B6 mice. For most of the experiments, one million of cells were injected, however in some experiments we used half-million due to technical reasons. For injection of dsRed-2D2 cells, 2×10^6 cells were used for better T cell visualization.

Clinical scoring of disease—Animals were monitored daily for development of experimental autoimmune encephalomyelitis according to the following criteria: 0, no disease; 1, limp tail; 2, hind limb weakness or partial paralysis; 3, complete hind limb paralysis; 4, front and hind limb paralysis; 5, moribund state. In addition, the body weight of the mice was measured during each clinical assessment⁵⁶. Researchers performing the clinical scoring were blinded to mouse identity.

Blood analysis—IL-6 and CXCL1 cytokines were measured in the serum using a multiplex inflammatory cytokine/chemokine kit (Meso Scale Discovery). Norepinephrine (NE) was measured using ELISA kit (Rocky Mountain Diagnostics).

Isolation of CNS-infiltrating cells—Recipient mice were euthanized 15 d after 2D2 cell transfer. Brain and spinal cords were removed and digested for 60 min with Liberase TL (Roche). Released cells were passed through a 70 μ m nylon mesh and subjected to density gradient centrifugation on a 30–70% Percoll gradient. Mononuclear cells found at the interface were harvested, washed extensively, and re-stimulated with PMA (10nM) and ionomycin (1 μ M) for 6h for assessment of cytokine production as described previously⁵⁶.

Bone marrow macrophage culture—To generate bone marrow-derived macrophages (BMM), bone marrow cells were plated in 10-cm bacteriological plastic plates with 10% FCS in RPMI 1640 medium supplemented with 50 ng/ml of recombinant mouse macrophage colony-stimulating factor (M-CSF). On day 7, adherent cells were collected and were replated at a density of one million cells/ml in 6-well tissue culture plates

RAW cell culture—RAW 264.7 were obtained from ATCC and mycoplasma absence was confirmed. Cells were cultured under 5% CO₂ in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ ml) and streptomycin (100 mg/ ml) at 37°C. For overexpression, cells were transfected with Nr4a1 cDNA expression vector (Origen) using Eugene kit (Promega). For knockdown, 0.5 μ g siRNA for Nr4a1 (Life Technologies) or CoREST (OriGene) was delivered by Eugene kit (Promega); for control, 0.5 μ g OriGene scrambled negative control siRNA was used.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously⁵⁷, with some changes. Briefly, cells were fixed for 10 min with 1% formaldehyde, lysed and sonicated in sonication buffer (0.1% SDS, 10mM Tris-HCl pH 8, 1mM EDTA supplemented with protease inhibitors and 5mM sodium butyrate) to produce ~500bp fragments, using Covaris system. Immunoprecipitation was performed overnight in sonication buffer supplemented with 1% Triton-20 and 150mM NaCl, using 5 µg of antibody for CoREST (Millipore) or acetylated histone H3 (Abcam). Recovered DNA was analyzed by qPCR with the primers corresponding to two proximal sites previously confirmed for Nr4a2 binding in the rodent TH promoter³⁷: NBS1 (F 5'-AGAGGATGCGCAGGAGGTAGGAG-3' and R 5'-GTCCCGAGTTCTGTCTCCAC-3'); NBS2 (F 5'-GGGGACTTGAAGACATCCAA-3' and R 5'-CCCAAGGGTTCATGTTAGGA-3') or with the primers corresponding to the proximal promoter of *Prl2B1* gene: F 5'-TCTGCATCCCAAAGTCCTTC-3' and R 5'-GTAACAGCCCCGAAACAAGA-3'.

Human PBMC isolation and analysis—Blood was obtained after informed consent from both healthy volunteers and patients at McGill University with recent onset of relapsing-remitting MS (RRMS) who had no exposure to systemic treatments in the prior 3 months. PBMC were isolated by Ficoll separation from the venous blood of healthy volunteers and patients with a recent onset of relapsing-remitting MS (RRMS) and with no exposure to systemic treatments in the prior 3 months, cryo-preserved and analyzed by flow cytometry. Lineage markers were CD3, CD19, CD66b.

Intravital microscopy

CX3CR1-GFP or CX3CR1-GFP/*Nr4a1*^{-/-} mice transferred with 2D2-DsRed T cells were anesthetized by intraperitoneal injection of ketamine and xylazine mixture (100–150 mg/kg and 15 mg/kg, respectively) and laid on a heating pad. The spinal cord at Th10–Th11 was surgically exposed as previously described^{58,59} and immobilized on a custom made stabilizing ring with a sealed 12-mm wide and 0.17-mm thick glass coverslip⁶⁰. To visualize neutrophils and blood volume 10 µg of anti-Ly6G antibody labeled with APC was injected intravenously through retro-orbital plexus. The imaging was performed on an upright resonant scanning Leica SP5 confocal microscope (Leica Microsystems) through a 25× water immersion objective (0.95NA, Leica Microsystems) mounted onto a piezoelectric nosepiece z-drive (Piezosystem Jena). GFP, DsRed and APC were imaged simultaneously with 488, 543 and 633 nm laser lines and stacks of 20–30 images with 0.714×0.714×2.5 µm voxel size were acquired. Image noise was reduced by line averaging during acquisition and median filtering (radius 1–2) post-acquisition. All image processing was performed in Fiji/ImageJ and SlideBook (3i) and the presented images were processed using identical procedures. Spectral spillover of DsRed to APC channel was removed by subtracting the DsRed channel from the APC channel. In addition, GFP spillover to the DsRed channel and background were further reduced by thresholding followed by contrast stretching.

Line scan analysis of 2D2 T cell infiltration was performed on maximum intensity projection images of spine parenchyma adjacent to the posterior spinal vein (sampled as 200 × 550 µm stripes). DsRed fluorescence intensity in lines perpendicular to the vein was

added, normalized to the wild-type day7 sample and represented as a line profile (2nd order smoothing with 50 neighbors, GraphPad Prism). The dendricity index ($= \text{Perimeter}^2 / \text{Area} \times 4\pi$, Baxter et al. 1991) of GFP⁺ cells in the spinal parenchyma was determined in maximum intensity projection images after GFP signal thresholding and mask creation. Perimeter and area mask statistics were extracted for the index calculation.

Flow cytometry

CNS-infiltrating cells were collected as described above. All samples were washed in Dulbecco's PBS (Gibco) with 2 mM EDTA and were stored on ice during staining and analysis. Cells (2×10^6 to 4×10^6) were resuspended in 100 μ l flow staining buffer [1% BSA (wt/vol) plus 0.1% (wt/vol) sodium azide in Dulbecco's PBS]. Fc γ receptors were blocked for 15 min and surface antigens on cells were stained for 30 min at 4 °C (CD3e clone 145-2C11 (BD Pharmingen); Ly6G clone 1A8 (Biolegend); Ly6C clone HK1.4 (Biolegend); CD11b clone M1/70 (BD Pharmingen); MHCII clone M5/114.15.2 (eBioscience); CD45 clone 30-F11 (Biolegend); MerTK clone BAF591 (R&D Systems) used with streptavidin-PE-Cy7 (405206, BioLegend); CD64 clone X54-517.1 (BD biosciences). LIVE/DEAD Fixable Dead Cell Stain (Life Technologies) was used for analysis of viability, and forward- and side-scatter parameters were used for exclusion of doublets from analysis.

For intracellular staining of TH, cells were fixed and made permeable with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Cells were stained for 30 min at room temperature with primary TH antibody (Millipore, clone LNC1) or isotype control antibody. Cells were stained for 30 min at 4 °C with secondary conjugated fluorescent secondary antibody (R-phycoerythrin F(ab')₂ Fragment of Goat Anti-Rabbit IgG (H+L), Life Technologies).

Calculations of percentages were based on live cells as determined by forward and side scatter and viability analysis. Mean fluorescence intensity was quantified, and expression was calculated relative to that of the wild-type control. Cellular fluorescence was assessed using LSR II or FACSaria II instruments (all from BD Biosciences) and data were analyzed with FlowJo software (TreeStar version 9.8).

Blood-brain barrier permeability analysis

Vascular permeability was evaluated by measurement of Evans blue dye (EBD) extravasation. EBD (30 mg/kg body weight, in 200 μ L) was injected intravenously in mice anesthetized with ketamine/xylazine and allowed to circulate for 30 minutes. The mice were then euthanized, the chest opened, the inferior vena cava transected, and the vasculature flushed with 20 mL saline via the right ventricle to remove excess intravascular dye. The brain and spinal cord was homogenized and incubated in 100% formamide at 37 °C for 24 hours to extract EBD. The concentration of EBD extracted was analyzed by spectrophotometry. Correction of optical densities (E) for contaminating heme pigments was performed as previously described, using the equation: $E_{620}(\text{corrected}) = E_{620} - (1.426 \times E_{740} + 0.03)^{61}$. Data were calculated as micrograms EBD per gram tissue.

Quantitative RT-PCR

RNA was isolated using RNeasy kit (Qiagen) and cDNA was prepared using Omniscript RT kit (Qiagen). For qPCR was performed on Light Cycler (Roche) using RT² primers (Qiagen) and Sybr-green dye (Roche). For Nr4a family genes, we used Taqman assay (Life Technologies).

Luciferase promoter-reporter assay—To generate TH-luciferase reporter, TH promoter (from –9 to –4555 bp) was cloned into pGL4.10(luc2) vector (Promega). For the assay, cells were seeded in 12-well plates; after overnight incubation, cells were transfected with 0.5 µg TH-luciferase reporter vector and 0.2 µg β-gal reporter vector (pCMVβ, Promega), and with other DNAs and siRNAs (see above) as indicated, using Fugene kit (Promega) for RAW cells and Amaxa Nucleofector system (Lonza) for BMM. After 24 hrs, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Fos expression vector was from Origen.

Co-immunoprecipitation (co-IP) and Western blot

RAW cells were transfected with Nr4a1 and samples were collected at 24h and 48h post transfection. Nr4a1 was precipitated using Dynabeads co-IP kit (Life Technologies) and the samples were analyzed by Western blot with CoREST antibody (Millipore).

Statistical Analyses

Data for all experiments were analyzed with Prism software (GraphPad). Unpaired *t*-tests and two-way analysis of variance were used for comparison of experimental groups. *P* values of less than 0.05 were considered significant. The data appeared to be normally distributed with similar standard deviation and error observed between and within experimental groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank K. Ley for helpful discussions, A. Rao for guidance in composing the manuscript, A. Crotti (UCSD) for guidance on microglia culture and D. Yoakum for assistance with mouse colony management. This work was supported by American Heart Association Scientist Development Grants #13SDG17060117 (to I.S.) and #12SDG12070005 (to R.N.H.), the La Jolla Institute Board of Directors Fellowship (to R.N.H.), Fondation Leducq Career Development Award and grants from the Sigrid Juselius Foundation and Academy of Finland (to M.U.K.), Pacific Northwest Udall Center P50-NS062684 (to M.D.), NIH R01 DK091183-21 (to C.K.G.), and NIH R01 HL118765 (to C.C.H.)

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Figure 1. Nr4a1 is highly expressed in myeloid cells in the CNS upon EAE onset

(a) *In vivo* imaging of Nr4a1 induction in spinal cord before (naïve) or 7 days after the transfer of 2 million 2D2-DsRed T cells into Nr4a1-GFP reporter mice; the images are representative of 3 independent experiments. The scale bar is 30 μ m. (b) Flow cytometry of leukocytes isolated from CNS of Nr4a1-GFP reporter mice at day 10 post EAE induction; a representative gating scheme is shown on the upper panel; quantification of Nr4a1-GFP expression in cells from 6 mice is on the bottom panel. MFI, median fluorescence intensity; error bars, s.e.m.

Figure 2. Nr4a1 loss leads to accelerated and exacerbated EAE

(a) Clinical EAE scoring, following transfer of 1 million Th1-polarized 2D2 T cells into WT or *Nr4a1*^{-/-} mice; *n*=5 in each group; the experiment was repeated at least 5 times and a typical result is shown. *p*<0.0001. (b) Absolute Nr4a1 mRNA expression in naïve mice of the indicated strains. Splenocytes were sorted to obtain monocytes, macrophages and granulocytes; brain cells were sorted to obtain microglia; mRNA was isolated from cell populations and Nr4a1 expression was analyzed using qRT-PCR. Four mice in each group were analyzed. (c) Clinical EAE scoring, following transfer of 1 million Th1-polarized 2D2

T cells into *Nr4a1^{fl/fl}* (WT) or *LysM-Cre;Nr4a1^{fl/fl}* (*Nr4a1^{LysM}*) recipients; *n*=5 in each group; data combined from two independent experiments. *p*<0.05. **(d)** Clinical EAE scoring, following transfer of half million Th1-polarized 2D2 T cells into *Nr4a1^{fl/fl}* (WT) or *Csf1r-Cre;Nr4a1^{fl/fl}* (*Nr4a1^{Csfr}*) recipients; *n*=5 in each group; data combined from two independent experiments. *p*<0.005. **(e)** Isolation and identification of leukocytes from CNS of *Nr4a1^{-/-}* or WT mice on the CX3CR1-GFP background, on day 7 post transfer of 2 million DsRed/2D2 T cells. On the left, a representative gating scheme. On the right, numbers of the indicated cell populations analyzed by flow cytometry, *n*=3. **(f)** 2 million DsRed-2D2 T cells were transferred into WT or *Nr4a1^{-/-}* mice on CX3CR1-GFP background, and Ly6G-APC was injected just before imaging to visualize neutrophils and blood vessels (blue). The scale bar is 50 μ m. Representative results of three separate experiments are showing *in vivo* confocal imaging of the spinal cord adjacent to the posterior spinal vein (PSV). **(g)** Blood concentrations of IL-6, CXCL1 and norepinephrine (NE) during onset of disease (day 7) in mice transferred with 1 million 2D2 T cells (*n*=3), or in naïve mice (*n*=4). n.d., non-detectable. **(h)** Gene expression level in total mRNA extracted from the lumbar spinal cord of WT or *Nr4a1^{-/-}* at different stages of EAE disease progression: 0, no disease; 1, limp tail paralysis; 2–3, hind limb weakness or partial paralysis; 3, complete hind limb paralysis; 4, front and hind limb paralysis; 5, moribund state; or in naïve mice. Three mice analyzed in each group. 2 way-ANOVA test in **(a,c,d)**; unpaired Student's *t*-test in **(e,g,h)**, *** *p*<0.001, ** *p*<0.01, * *p*<0.05; error bars, s.e.m.

Figure 3. NE-producing macrophages have an essential role in EAE

(a–b) One million 2D2 cells were transferred into WT or *Nr4a1*^{-/-} mice, which were untreated or treated daily with 20 mg/kg of the α1 blocker prazosin (a) or 100 mg/kg of the noradrenergic toxin 6-OHDA (b) and disease progression was scored. *n*=5 in each group, representative experiments out of two for each treatment are shown. 2 way-ANOVA test; error bars, s.e.m. In (a), *p*<0.01 for WT and *p*<0.05 for *Nr4a1*^{-/-} mice; in (b), *p*<0.01 for both strains. (c) Isolation and identification of wild-type CNS-infiltrating TH⁺ immune cells following 0.5 million 2D2 transfer. Gating scheme is as in Fig. 1b. On the right, quantification of 3 separate experiments. (d) One million 2D2 cells were transferred into WT mice transplanted with either WT or dopamine-deficient floxed stop (DDFs) bone marrow and disease progression was scored, *n*=6. *p*<0.01. (e) Half million 2D2 cells were transferred into WT or *Th*^{LysM} mice and disease progression was scored, *n*=8; data combined from two independent experiments. *p*<0.005. (f) IL-6 mRNA expression in BMM, untreated (CTL) or treated with 1μM NE in the presence or absence of NE receptor blockers (α1-blocker, prazosin and β1-blocker, atenolol, 1μM each), and in IFN-γ-treated BMM in the presence or absence of TH inhibitor AMPT (100μM). Expression relative to CTL BMM is shown. Different letters denote statistically different groups as by one-way ANOVA multiple comparisons. (g) Levels of mRNA encoding IL-6 and IL-6 associated chemokines in IFN-γ-treated BMM from WT or DDFs mice. Expression relative to corresponding values in untreated BMM is shown. Data in (f,g) are from triplicates representative out of 3 independent experiments. 2 way-ANOVA test in (a,b,d,e); unpaired Student's *t*-test in (c,g), *** *p*<0.001, ** *p*<0.01, * *p*<0.05; error bars, s.e.m.

Figure 4. Nr4a1 directly suppresses TH expression in macrophages

(a) *Th* mRNA expression in control BMM and IFN- γ -treated BMM, isolated from WT or *Nr4a1*^{-/-} mice; data are from triplicates representative of 2 separate experiments. (b) Western blot showing TH protein in untreated (CTL) or IFN- γ -treated BMM; representative of two independent experiments. (c) *Th* mRNA expression following knockdown (KD) or overexpression (OE) of Nr4a1 as well as CoREST knockdown in RAW macrophages; data are from triplicates representative of 3 independent experiments; each sample is normalized to the average of the corresponding control (CTL) (empty vector for OE and control siRNA for KD) and the controls are pooled. (d) Analysis of the interaction of Nr4a1 and CoREST. Co-IP was performed in RAW cells with anti-Nr4a1 antibody and Western blots were developed with anti-CoREST antibody. RAW cells were transfected with Nr4a1 and samples were collected at 24hrs and 48hrs post transfection; representative of two independent experiments. (e) ChIP analysis was performed in RAW cells with either anti-CoREST or anti-acetyl-H3 antibodies; the immunoprecipitated DNA was analyzed by qPCR with the primers for indicated regions; enrichment was calculated relative to the control region (Pr12B

gene promoter). Data are average of 3 separate experiments. On the upper panel, *Th* promoter is shown with two proximal Nr4a family binding sites (NBS). Unpaired Student's *t*-test in (a,c), ** $p<0.01$, * $p<0.05$; error bars, s.e.m.

Figure 5. TH protein abundance is increased in the monocytes of MS patients

Frozen PBMC were derived from relapsing-remitting MS (RRMS) patients and healthy donor controls from McGill University and analyzed by flow cytometry. Researchers performing the experiment and analyzing the data were blinded to sample identity. **(a)** Gating strategy. Monocytes were identified as lineage (CD3, CD19, CD66b) negative, HLA-DR⁺, CD86⁺, and CD14⁺. **(b)** Comparison of TH expression in monocytes vs. lineage positive cells in a representative RRMS patient. **(c)** Comparison of TH expression in CD14⁺ monocytes isolated from healthy controls and RRMS patients. * $p < 0.05$; unpaired Student's *t*-test. Researchers performing the experiment and analyzing the data were blinded to sample identity.