Stimulation of the melanocortin-1 receptor leads to direct neuroprotection via orphan nuclear 4 receptor in inflammatory neurodegeneration

One sentence summary: The recently approved drug NDP-MSH efficiently and sustainably ameliorates ongoing progressive inflammatory neurodegeneration in mice as well as human neurons by combining anti-inflammatory and direct neuroprotective effects.

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ABSTRACT
In inflammation-associated progressive neuroinflammatory disorders, e.g. multiple sclerosis (MS), inflammatory infiltrates, including Th1 and Th17 cells, cause demyelination and axonal/neuronal degeneration. Regulatory T-cells (Treg) have evolved to control the activation and infiltration of autoreactive T-cells into the CNS. However, in experimental autoimmune encephalomyelitis (EAE) and MS, Treg function is severely impaired. We show that Nle4-D-Phe7-α-melanocyte-stimulating hormone (NDP-MSH), a melanotropic peptide derived from α-melanocyte-stimulating hormone, and a known potent immunomodulator, induces functional Treg, which inhibit EAE progression. Strikingly, NDP-MSH also prevented immune cell infiltration into the CNS by restoring the integrity of the blood-brain barrier and exerted strong, long-lasting direct neuroprotective effects in a spontaneous EAE model, prevented excitotoxic neuronal cell death, and re-established action potential firing. Neuroprotection by NDP-MSH was mediated via signaling through melanocortin-1 and orphan nuclear 4 receptors in mouse and human neurons. NDP-MSH has recently received European Medicines Agency approval for the treatment of erythropoietic porphyria and our novel data strongly support its use in treating neuroinflammatory diseases, e.g. relapsing-remitting MS.
INTRODUCTION

Neuroinflammatory disorders, such as multiple sclerosis (MS) or Devic's disease are characterized by an immune-mediated, chronic inflammatory and demyelinating disease of the central nervous system (CNS) leading to severe disability, and have an estimated prevalence of over 2.5 million cases worldwide ((1); www.nationalmssociety.org). They are known to be mediated by peripherally generated autoreactive T-cells infiltrating into the CNS, where they are re-stimulated by antigen-presenting cells, resulting in local expansion of pathogenic Th1 and Th17 cells, tissue damage to oligodendrocytes, and subsequently, a secondary infiltration of bystander immune cells, controlling the disease's perpetuation (2, 3). Regulatory T-cells (Treg) are involved in limiting the activation, proliferation and infiltration of pathogenic effector cells into the CNS (1, 4). However, in MS and its murine model of experimental autoimmune encephalomyelitis (EAE), an altered distribution, modified phenotype, and, particularly, impaired suppressive activity of Treg have been associated with disease progression (5-7). Accordingly, immunomodulatory drugs known to induce anti-inflammatory pathways and functional Treg, such as glatiramer acetate, fingolimod, or interferon β, at least partially ameliorated disease in MS and EAE by up-regulating Treg numbers or increasing their suppressor function (8-10). Although these drugs attenuated pro-inflammatory T-cell responses, there is doubt that they directly protect neurons from inflammatory cell death (11, 12). Consequently, in a model of opticospinal encephalomyelitis involving myelin-oligodendrocyte-glycoprotein (MOG)-specific T- and B-cells, glatiramer acetate treatment only resulted in a nonsignificant trend towards lower disease activity (13).

The neuropeptide α-melanocyte-stimulating hormone (α-MSH) is a known mediator of skin pigmentation, but it also acts as potent immunomodulator capable of expanding thymic-derived and peripherally induced Treg, which inhibit the proliferation of psoriatic mouse as well as human Th1 and Th17 cells (14). The biologic effects of α-MSH are mediated by binding to one of five G-protein-coupled melanocortin receptors (termed Mc1r–Mc5r) (15, 16). All five melanocortin receptors are highly expressed on neuronal cells in the CNS, and particularly Mc1r, which has been shown to mediate the immunomodulatory effects of α-MSH, can additionally be found on immune cells (14, 16, 17). Mc1r is functionally coupled to adenylyl cyclase. Hence, the majority
of Mc1r-mediated effects of α-MSH are based on the activation of cyclic adenosine monophosphate (cAMP)-dependent signaling pathways. These include the activation of protein kinase A (PKA), the phosphorylation of cAMP response element binding protein (CREB), the reduction of nuclear factor of activated T-cells (NFAT) activity or the suppression of nuclear factor (NF)-κB translocation through the protection of IκBα from phosphorylation (18, 19). Under physiologic conditions, cells of the CNS are a source of α-MSH, and in vitro studies suggest a possible neuroprotective role since the melanocortin inhibited NF-κB activation in tumor necrosis factor α-activated rat Schwann cells or lipopolysaccharide-activated human glioma cells (20, 21). However, α-MSH is chemically unstable, highly sensitive to proteolytic degradation, and undetectable in the plasma 30–90 minutes after intravenous injection (22). Accordingly, the neuropeptide has been modified at the amino acids in positions 4 and 7 resulting in the protease-stable Nle⁴-D-Phe⁷-α-MSH (NDP-MSH), which, in addition to its improved stability, is characterized by an increased affinity to Mc1r (23, 24). NDP-MSH (Scenesse®) has recently been approved by the European Medicines Agency (EMA) for the treatment of erythropoietic porphyria, an inherited disease characterized by deficiencies in heme biosynthesis and leading to painful photosensitivity as well as liver failure.

Here we show that NDP-MSH induced functional Treg able to efficiently suppress pathogenic Th1 and Th17 cells in ongoing active as well as passive EAE. Strikingly, in addition, NDP-MSH restored the integrity of the blood-brain barrier (BBB) and mediated a strong and long-lasting neuroprotective effect, which was demonstrated in vivo and in vitro by an unexpected mechanism of action.
RESULTS

**MSH, by binding to Mc1r, ameliorates ongoing EAE**

To characterize the impact of melanocortins on the development of inflammatory neurodegeneration C57BL/6 (wild-type) mice were immunized with MOG peptide and systemically treated with $\alpha$-MSH in 48-h intervals. $\alpha$-MSH completely protected mice from developing clinical signs, which was associated with reduced numbers of inflammatory foci and decreased CNS demyelination (Fig. 1, A, B and fig. S1). In skin inflammation $\alpha$-MSH exerted its anti-inflammatory effect by expanding Treg, finally resulting in the inhibition of effector T-cell activation (14). In line with these data, Th1 and Th17 cells were significantly reduced in the CNS and draining cervical lymph nodes from $\alpha$-MSH-treated mice compared with phosphate-buffered saline (PBS)-injected controls whereas we detected similar levels of both cell subsets in the spleen (Fig. 2C and figs. S2A and S2B). Upon co-culture with MOG-loaded dendritic cells (DC), total CD4$^+$ T cells isolated from the CNS of $\alpha$-MSH-treated mice showed a reduced antigen-specific proliferation versus CD4$^+$ T cells from PBS-treated controls (fig. S2C), pointing to the $\alpha$-MSH-mediated induction of Treg in inflammatory neurodegeneration, which was confirmed by flow cytometry, histology and gene expression studies (Fig. 1, D, E and figs. S3A, S3B, S3C, and S3D). Mouse CD4$^+$ T cells do not express Mcr (25), thus $\alpha$-MSH exerted its immunomodulatory capacities in an indirect mechanism by inducing tolerogenic DC (Fig. 1F and fig. S3E). Worth mentioning, the beneficial effect of $\alpha$-MSH was mediated by signaling through Mc1r since treatment of mice deficient for Mc1r or injection of wild-type mice with the C-terminal tripeptide of $\alpha$-MSH (KPV), which is known to have similar anti-inflammatory capacities but lacks the Mc1r binding motif (15), did not abrogate disease development (Fig. 1, G, H and figs. S4A, S4B, S4C, and S4D).

Under physiologic conditions, $\alpha$-MSH is synthesized directly in the CNS (axons expressing $\alpha$-MSH are detectable in various cortical areas, but especially in the limbic cortical regions) and rapidly degraded (22). Hence, melanocortins resistant to enzymatic cleavage, such as NDP-MSH, have been generated and since NDP-MSH (Scenesse®) has recently been EMA-approved, we
investigated whether NDP-MSH might be able to ameliorate ongoing EAE. Therefore, mice were immunized with MOG peptide and after onset of paralysis (clinical score ≥2) NDP-MSH was injected in 48-h intervals. Strikingly, already after the third injection, NDP-MSH recipient mice went into complete remission, whereas PBS-treated mice developed paraparesis. This was associated with markedly decreased numbers of pathogenic Th1 and Th17 effector T-cells as well as up-regulated levels of Treg in the CNS of NDP-MSH- compared with PBS-treated mice (Fig. 2, A, B, C, D, and E). Similar to α-MSH also NDP-MSH exerted its biological effects by inducing tolerogenic DC (figs. S5A, S5B, and S5C). Of note, the anti-inflammatory capacity of NDP-MSH was increased compared with α-MSH and similar to α-MSH, dependent on signaling through a functional Mc1r (Fig. 2, A, B, C, D, and E and figs. S6A, S6B, S6C, and S6D), which was most likely due to the improved stability and affinity to Mc1r. In NDP-MSH-treated mice, reduction of disease severity was paralleled by decreased numbers of inflammatory lesions containing mononuclear cell infiltrates, less demyelinated areas, and a significantly down-regulated expression of rab interactive lysosomal protein-like 2 (RILPL2) or amyloid precursor protein (APP), markers known to correlate with CNS inflammation and neurodegeneration, in the CNS (Fig. 2, D, E). In vivo magnetic resonance imaging (MRI) from MOG-immunized mice 6 days after the first injection of NDP-MSH or PBS revealed striking alterations in periventricular signal enhancement due to contrast agent (gadolinium-diethylenetriamine pentaacetic acid; Gd-DTPA) accumulation between NDP-MSH- and PBS-treated mice. These observations point to an improved integrity of the endothelial/parenchymal basement membrane in NDP-MSH-treated mice and suggested BBB leakage in control animals (Fig. 2F). We have previously shown that BBB integrity and T-cell extravasation in EAE are controlled by endothelial basement membrane laminins, whereby laminin-α5 (Lama5), which is inhomogeneously expressed during CNS inflammation, hampers leukocyte infiltration into the brain (26). Therefore, immunofluorescence staining of EAE CNS tissue using an antibody to Lama5 was performed. Surprisingly, a uniform expression of Lama5 was detectable in the basement membrane of NDP-MSH-treated mice, whereas PBS-treated controls showed the expected patchy staining (Fig. 2G). Moreover, extravasation of pathogenic effector T-cells, as evidenced by staining for CD4 and interleukin (IL)-
17, was only detectable in PBS-treated mice at sites lacking Lama5 (Fig. 2G). To further confirm an improved BBB integrity upon NDP-MSH treatment, mice were intravenously injected with fluorescein isothiocyanate (FITC)-labeled dextran particles and the extravasation of the beads into the CNS was quantified. Interestingly, numbers of dextran particles were significantly reduced in the spinal cord from NDP-MSH-treated mice compared with controls (Fig. 2H) again strengthening the hypothesis that NDP-MSH restores BBB function during CNS inflammation. Since pertussis toxin is known to permeabilize endothelial barriers (27) and, moreover, the administration of complete Freund’s adjuvant might up-regulate Toll-like receptor signaling, thus potentially resulting in the activation of immune cells, we intended to overcome these limitations of MOG-induced EAE by investigating the effects of NDP-MSH in a second, independent, spontaneous model of inflammatory/demyelinating diseases of the CNS. Hence, TCRMOG x IgHMOG mice (Devic) (28) were treated with NDP-MSH starting at a clinical score of ≥2. Devic mice develop ascending paralysis 5–6 weeks after birth and reach disease maximum at the age of 40-50 days, as evident from the PBS-treated controls (Fig. 3A). Notably, mice injected with NDP-MSH had a significantly lower cumulative score and were almost completely in remission after 28 days of treatment, whereas PBS-treated controls had to be sacrificed at the age of 7 weeks due to animal welfare regulations (Fig. 3A). In addition to clinical signs, NDP-MSH markedly reduced the infiltration of total CD4+ T-cells into the spinal cord as well as pathogenic Th1 and Th17 cells in the periphery (Fig. 3, B, C), which resulted in less inflammatory foci, demyelinated areas, and a significantly down-regulated expression of neuronal/axonal damage markers compared with controls (Fig. 3D). Interestingly, also in mice spontaneously developing a Devic-like disease, NDP-MSH induced a uniform expression of Lama5 in the basement membrane, most likely accounting for the reduced T-cell infiltration into the CNS of treated mice versus controls (Fig. 3E). Next, we characterized the duration of the NDP-MSH-mediated protective effect in Devic mice. Therefore, treatment was terminated at day 64 when mice were in remission, and animals were monitored for disease relapse up to 5 months. As depicted in Fig. 3F the beneficial effect of NDP-MSH persisted for at least 60 days without any treatment, and we did not observe alterations in the clinical score until day 124. Thereafter mice started to develop
ascending paralysis (Fig. 3F), indicating that 14 initial intravenous injections of NDP-MSH from days 36 to 64 were sufficient to keep Devic mice in remission for more than 8 weeks before recurrence of disease activity. Of note, NDP-MSH-treated Devic mice in remission (day 124) showed less demyelinated areas in the CNS, a uniform expression of Lama5 in the basement membrane, and a significantly reduced infiltration of Th1 and Th17 cells into the spinal cord compared with NDP-MSH-treated Devic mice after recurrence of disease activity (day 194; Fig. 3, G, H).

The NDP-MSH-mediated expansion of Treg or induction of tolerogenic DC cannot account for long-lasting (neuro-) protective effects

To analyze whether the generation of tolerogenic DC and induction of Treg indeed explained the beneficial effect of NDP-MSH in vivo, we depleted Treg in DEREG mice (29) and DC in CD11c.DOG mice (30) and subsequently induced EAE (the efficiency of cell depletion is depicted in figs. S7A, S7B, S7C, and S7D). However, mice lacking Treg or DC were still protected from disease progression upon NDP-MSH injection (Fig. 4, A, B, C, D, E, and F), clearly indicating an additional mechanism of NDP-MSH action besides inducing immunosuppressive cell subsets. This hypothesis was further strengthened by the observations in Devic mice, where it was difficult to explain the long-lasting effect of NDP-MSH (even in the absence of the drug, Fig. 3F) exclusively by the expansion of Treg or tolerogenic DC. Thus, our data pointed to a direct neuroprotective effect of NDP-MSH in vivo.

NDP-MSH induces neuroprotection and restores action potential (AP) generation by up-regulating the orphan nuclear receptor Nr4a1

To identify mechanisms associated with NDP-MSH-mediated neuroprotection, we performed gene expression studies in CNS tissue from MOG-immunized, NDP-MSH- or PBS-treated mice. Surprisingly, upon NDP-MSH treatment, we detected the regulation of gene-clusters linked to immune responses in C57BL/6J<sup>e<sup>e</sup></sup> mice, whereas in wild-type mice, the regulated gene-clusters were linked to pathways associated with neuropeptide/neurotransmitter receptor activity, nervous
system development or neuron fate (figs. S8A, S8B, and S8C). In the most prominent gene-cluster upregulated upon NDP-MSH treatment in the CNS from MOG-immunized wild-type mice, we identified the orphan nuclear receptor Nr4a1 closely related to melanocortin receptors (Mc1r, Mc5r) or the neuropeptides somatostatin, neuropeptide Y, and vasoactive intestinal peptide (fig. S8A). Nr4a1 has been shown to be induced after excitotoxic stress in neurons and to promote neuronal survival (31). Moreover, in melanocytes, Mc1r signaling induced the expression of Nr4a1 upon cellular stress (32, 33). These data, together with our gene-expression studies, suggested Nr4a1 as a potential mediator of NDP-MSH-mediated neuroprotection. Interestingly, Mc1r was required for the expression of Nr4a1 in brain tissue from MOG-immunized mice and in isolated neurons under steady-state as well as excitotoxic conditions (figs. S9A, S9B, and S9C), thus supporting a functional link between Mc1r and Nr4a1. To investigate the effect of NDP-MSH on neuronal survival and the interaction of Mc1r and Nr4a1 signaling during neuroprotection in more detail, purified hippocampal neurons were treated with glutamate, known to cause neuronal cell death (34), in the absence or presence of NDP-MSH. Notably, in glutamate-treated wild-type neurons, the neuropeptide up-regulated both receptors, Mc1r and Nr4a1, and blocked the expression of the pro-apoptotic caspases 3 and 8 (Fig. 5, A, B). In contrast, we did not observe the induction of either receptor or a significant down-regulation of pro-apoptotic caspases in glutamate-stimulated neurons from C57BL/6J e/e mutants (Fig. 5, A, B) indicating that Mc1r signaling is essential for the prevention of neuronal damage via up-regulation of Nr4a1. Next, we analyzed the signaling pathway linking Mc1r and Nr4a1 in neurons. Since Mc1r is known to exert its downstream effects by phosphorylation of CREB or p38 mitogen-activated protein kinase (MAPK) and Nr4a1 can be activated by either pathway (16, 35, 36), we quantified phospho-CREB and phospho-p38 MAPK in neurons from wild-type and C57BL/6J e/e mice. Interestingly, Mc1r ligation by NDP-MSH resulted in a significant increase of phospho-CREB, whereas the neuropeptide did not modulate the levels of phospho-p38 MAPK (Fig. 5C), indicating that the direct protective effect of NDP-MSH was mediated by binding to Mc1r, phosphorylation of CREB and subsequent activation of Nr4a1.
Next, we questioned if NDP-MSH is able to preserve functional neuronal integrity under glutamate-mediated excitotoxic conditions. Using whole-cell patch-clamp recordings in the current-clamp mode, we detected a resting membrane potential of $-53 \pm 1.3$ mV or $-50 \pm 1.1$ mV; $n = 25$, $P = 0.171$ for untreated or NDP-MSH-treated cultured hippocampal neurons, respectively (Fig. 5, D, E). This was accompanied by regular AP generation in untreated neurons triggered by a depolarizing current step (Fig. 5E), while NDP-MSH treatment resulted in an increased number of fired spikes. Application of 10 $\mu$M glutamate induced a massive depolarization in untreated hippocampal neurons towards $-31.11 \pm 2.12$ mV ($n = 22$, $P = 0.001$) resulting in a depolarization block without any AP generation. Pretreatment with NDP-MSH significantly prevented glutamate-induced depolarization ($40.74 \pm 1.568$ mV; $n = 30$, $P = 0.0005$) allowing all recorded neurons to respond to the current stimulus with AP generation, although AP numbers, width, and height differed from control conditions (Fig. 5, D, E and figs. S9D, S9E, and S9F). Importantly, this direct neuroprotective effect of NDP-MSH was neither detectable in neurons lacking a functional Mc1r (Fig. 5, F, G) nor Nr4a1 (Fig. 5, H, I), again endorsing that binding of NDP-MSH to Mc1r and signaling via Nr4a1 is essential for the neuroprotective effect of NDP-MSH.

**Nr4a1 deficiency abolished the beneficial effect of NDP-MSH**

To investigate, whether the neuroprotective effect of NDP-MSH was indeed mediated by Nr4a1 signaling in vivo, *Nr4a1* deficient mice (Nr4a1$^{+/+}$) (37) were immunized and treated with NDP-MSH. Notably, mean clinical scores and neuronal damage, as evidenced by demyelination and RILPL2 or APP expression, were similar in NDP-MSH-treated Nr4a1$^{+/+}$ mice and PBS-injected controls, although NDP-MSH-treated animals had increased numbers of Treg and down-regulated levels of pathogenic Th1 and Th17 cells in the CNS (Fig. 6, A, B, C, and D), thus clearly indicating that Nr4a1 deficiency abolished the beneficial effect of NDP-MSH. Additionally, in the absence of Nr4a1, NDP-MSH was not able to improve the BBB integrity as indicated by a patchy Lama5 expression and the infiltration of pathogenic Th17 cells into the CNS at sites with low or absent Lama5 (Fig. 6E) in NDP-MSH-treated Nr4a1$^{+/+}$ mice.
To clearly distinguish the neuroprotective effect of NDP-MSH from its anti-inflammatory capacities, we generated bone marrow chimeric mice by transferring wild-type or \( \text{Nr4a1}^{-/-} \) bone marrow cells into lethally irradiated wild-type or \( \text{Nr4a1}^{-/-} \) recipients. After having confirmed bone marrow reconstitution (fig. S10A), EAE was induced in chimeric mice. Notably, NDP-MSH entirely ameliorated ongoing EAE in wild-type recipients of \( \text{Nr4a1}^{-/-} \) bone marrow, whereas in \( \text{Nr4a1}^{-/-} \) recipients of wild-type bone marrow the neuropeptide had a mild, but nevertheless significant effect, indicating that indeed both the anti-inflammatory and neuroprotective properties of NDP-MSH are crucial (Fig. 6F). However, the neuroprotective effect of NDP-MSH seemed to be of major importance compared with the anti-inflammatory capacity during amelioration of CNS inflammation and degeneration, since we observed complete remission in chimeric mice with neurons of wild-type, but only partial remission in chimeric mice with neurons of \( \text{Nr4a1}^{-/-} \) origin (Fig. 6F and fig. S10B). In support of this, MOG-immunized chimeric mice with neurons of wild-type and immune cells of \( \text{Nr4a1}^{-/-} \) origin showed almost no demyelinated areas in the CNS upon NDP-MSH treatment and exhibited a continuous Lama5 expression in the basement membrane, preventing the extravasation of pathogenic effector cells into the CNS. In contrast, we observed demyelination and a moderate immune cell infiltrate in the CNS of \( \text{Nr4a1}^{-/-} \) recipients of wild-type bone marrow (Fig. 6, G, H and fig. S10C). To identify non-hematopoietic cells in the brain, besides neurons, expressing \( \text{Nr4a1} \) immunofluorescence staining was performed demonstrating the presence of the nuclear orphan receptor in astrocytes, oligodendrocytes and microglia (fig. S11).

**NDP-MSH protected human neuronal cells from glutamate-induced apoptosis**

Next, we characterized the neuroprotective effect of NDP-MSH in human neuronal cells. Therefore, human neurons were differentiated from the ReNcell VM progenitor cell line (38) and treated with glutamate in the absence or presence of NDP-MSH. Similar to murine cells, human neurons expressed MC1R and up-regulated NR4A1 on mRNA and protein level in response to NDP-MSH (Fig. 7, A, B). Moreover, in NDP-MSH-treated cells, we observed a significant down-regulation of the pro-apoptotic caspase 3 after glutamate-induced excitotoxicity compared with PBS-treated controls (Fig. 7B), suggesting that MC1R/NR4A1 signaling plays a critical role in
neuroprotection and the prevention of apoptosis in human neurons as well. Finally, we assessed whether MC1R/NR4A1 interactions might be involved in the development or progression of MS. Therefore, the expression of MC1R and NR4A1 was quantified in human brain tissue from individuals with MS. Strikingly, the expression of MC1R was dramatically reduced in NEUN* neurons from MS brains compared with controls (Fig. 7C), thus resulting in significantly decreased numbers of neurons expressing both, NR4A1 and MC1R. Hence, our data might suggest that in MS, due to the reduced expression of MC1R, melanocortins might not be able to exert sufficient neuroprotective effects, thus resulting in increased damage, demyelination, and immune cell infiltration into the CNS.
DISCUSSION

Here, we conclusively demonstrated that NDP-MSH efficiently inhibited ongoing inflammation-associated and progressive degenerative disorders of the CNS. This effect was mediated by a combination of the potent anti-inflammatory and immunomodulatory capacities of NDP-MSH with the NDP-MSH-induced long-lasting neuroprotection and repair of BBB leakage (Fig. 8, A, B). On a cellular level, NDP-MSH, by binding to Mc1r, prevented excitotoxic cell death in murine as well as human neurons and partially restored their function. Mechanistically, Mc1r ligation resulted in the downstream phosphorylation of CREB and subsequent CREB-dependent activation of Nr4a1, leading to the induction of neuroprotective genes, and presumably also the up-regulation of Lama5 (Fig. 8B).

Targeting melanocortin receptors to control peripheral inflammation has frequently been used. Accordingly, the administration of α-MSH to mice or rats with psoriasis, colitis, rheumatoid arthritis, or infections resulted in a marked amelioration of disease mediated by the suppression of pathogenic effector cells, the down-regulation of pro-inflammatory and the induction of anti-inflammatory cytokines (14, 39-41). Since some immunomodulatory properties of the melanocortin system, including the induction of functional Treg (14), might be relevant to CNS inflammation as well, we assessed the therapeutic potential of NDP-MSH, a melanocortin peptide recently approved for the treatment of certain skin diseases, during established inflammatory and degenerative CNS disorders. Surprisingly, NDP-MSH induced complete remission in MOG-induced EAE as well as in Devic’s disease even after a couple of intravenous injections. This observation was in stark contrast to previous studies showing a 24-h delay in disease onset and a 50% decreased disease severity after daily oral administration of α-MSH starting 7 days before MOG immunization or a reduction in clinical score from 2.1 to 1.3 after α-MSH treatment of proteolipid protein-immunized mice (42, 43). However, the mild effects of α-MSH (42, 43) might be attributed to its chemical instability. To overcome this problem, mice with proteolipid protein-induced EAE were treated with Ac-SVα-MSH, an α-MSH analog, in which the amino acids Tyr, Ser and Phe in positions 2, 3, and 7 were replaced by Ser, Ile, and D-Phe to increase the stability and affinity to Mc1r as well as Mc5r. But only the pre-emptive administration starting at the day of
immunization resulted in a reduction of disease severity, whereas Ac-SVα-MSH injected after onset of paralysis had no effect (18). Since we have clearly shown that the protective effect in inflammation-associated CNS diseases is mediated by ligation of Mc1r (Fig. 1G and Fig. 8B), the different efficacy of Ac-SVα-MSH and NDP-MSH might be explained by the specificity of NDP-MSH for Mc1r, whereas Ac-SVα-MSH binds to Mc1r and Mc5r with similar affinity (18, 23, 24). However, it is highly unlikely to explain the tremendous and long-lasting beneficial effect of NDP-MSH, particularly in Devic’s disease (>8 weeks of remission without any treatment), just by the induction of Treg. In line with this, by depleting Treg, performing gene expression studies, conducting functional assays in isolated neurons, and generating bone marrow chimeric mice, we identified a strong and direct neuroprotective potential of NDP-MSH in inflammation-associated and progressive degenerative CNS diseases. The ability of melanocortins to reduce neuronal damage after traumatic brain injury or counteract cognitive decline in mice with Alzheimer’s disease has already been discussed (44, 45); however, the underlying molecular mechanisms have not yet been elucidated. Here, we clearly demonstrated that NDP-MSH induces neuroprotection by binding to Mc1r, resulting in subsequent activation of Nr4a1 via phosphorylation of CREB (Fig. 5C and Fig. 8B). Nr4a1 belongs to a family of orphan nuclear receptors for which, thus far, no physiologic ligand has been identified (46). In mice or humans, this family consists of three receptors: Nr4a1 (also known as Nur77), Nr4a2 (Nurr1) and Nr4a3 (Nor-1). All three Nr4a receptors are involved in basic cellular processes like proliferation, apoptosis, differentiation and stress responses (46). Nr4a2 seems to be crucial for the development of dopaminergic neurons as concluded from Nr4a2–/– mice presenting with severe defects in brain architecture (47). Additionally, in EAE the activation of Nr4a2 with isoxazolopyridinone 7e significantly reduced disease severity and was suggested to attenuate neurodegeneration (48). Nr4a1 has been implicated in neuroprotection since this receptor was up-regulated upon excitotoxic and oxidative stress in hippocampal neurons and mediated the induction of neuroprotective genes, such as Abl2, Prkaa2, or Sod1 (31). Notably, in melanocytes, Nr4a1 expression can be regulated in response to Mc1r signaling (33). In line with these observations, upon NDP-MSH treatment we were unable to detect Nr4a1 in hippocampal neurons,
from C57BL/6J\textsuperscript{e}e mice lacking a functional Mc1r. Whereas we have shown the restoration of AP firing in neurons, Yin \textit{et al.} demonstrated an augmented DNA repair in UV-irradiated keratinocytes after ligation of Mc1r and induction of Nr4a1 (32), thus suggesting a general protective mechanism of this pathway.

Experiments in bone marrow chimeric mice clearly revealed that a combination of the anti-inflammatory and neuroprotective properties of NDP-MSH is essential to induce the observed long-lasting beneficial effect in inflammation-associated and degenerative diseases of the CNS. In this context, a critical hallmark of NDP-MSH might be the restoration of the BBB integrity, which we detected in active EAE as well as in Devic’s disease. Besides immune cells or neurons, Mc1r is expressed on endothelial cells, including brain endothelial cells, and ligation of Mc1r protected against endothelial dysfunction (47, 49). Importantly, this protective effect was abrogated in C57BL/6J\textsuperscript{e}e mice, which showed an increased arterial stiffness and susceptibility to inflammation-driven vascular dysfunction (50). Besides Mc1r, also Nr4a1 is known to be rapidly induced in endothelial cells upon pro-inflammatory stimuli and to mediate cell survival, whereas the knock-down induced endothelial cell apoptosis (51). Furthermore, it has been shown that Nr4a1 overexpression led to decreased monocyte adhesion to endothelial cells (52). Additionally, \(\alpha\)-MSH by binding to melanocortin receptors inhibited the transmigration of neutrophils across an endothelium–epithelium bilayer under inflammatory conditions (53). Hence, it is conceivable, that Mc1r and Nr4a1, besides inducing direct neuroprotection, also cooperate in improving the function of the BBB during CNS inflammation by protecting endothelial cells from damage and preventing leukocyte transmigration. A characteristic hallmark of an impaired BBB integrity during CNS inflammation is the inhomogeneous expression of Lama5. We have previously demonstrated that basement membrane laminins control leukocyte extravasation into the CNS, whereas Lama5 hampers the migration, thus enabling leukocyte infiltration into the brain only in areas with little or no Lama5 expression (26). Interestingly, NDP-MSH induced a continuous Lama5 expression in the basement membrane, thereby improving BBB integrity and preventing leukocyte extravasation. These observations might suggest that in endothelial basement membranes of the CNS, the protective synergistic effect of Mc1r and Nr4a1 signaling could,
besides preventing damage of endothelial cells, also include the induction of Lama5, thus conferring an impermeability to the BBB. Alternatively, the anti-inflammatory effects of NDP-MSH could have contributed to the improvement of BBB integrity.

Together, beside anti-inflammatory and immunomodulatory capacities we detected a strong and direct neuroprotective effect of NDP-MSH in mouse as well as human neurons, which, in combination with the restoration of the BBB integrity and reduced leukocyte infiltration into the brain, led to long-lasting CNS-protection. Since NDP-MSH has recently been EMA-approved for the treatment of erythropoietic porphyria, our novel data strongly suggest this drug could be used for the treatment of inflammation-associated degenerative CNS diseases, such as relapsing-remitting MS. Under physiologic conditions, melanocortins, such as $\alpha$-MSH, are expressed in the brain (19-21). However, as we have shown for the first time, Mc1r expression is significantly downregulated in MS compared with healthy human brain (Fig. 7C). Thus, it is conceivable that the naturally occurring $\alpha$-MSH, which is characterized by a lower affinity to Mc1r compared with the synthetic NDP-MSH, might not be potent enough to induce neuroprotection and BBB repair in individuals with MS. This could potentially be overcome by NDP-MSH treatment.
MATERIAL AND METHODS

Experimental Design

The aim of this study was to characterize the therapeutic potential of the recently approved drug NDP-MSH (Scenesse®) in progressive neuroinflammatory diseases. Therefore, mouse models of active (MOG-induced) and passive EAE (Devic's disease) were used revealing an anti-inflammatory as well as long-lasting neuroprotective effect of NDP-MSH even in the absence of the drug. To elucidate the underlying molecular mechanism gene expression profiling using Affymetrix Mouse Gene 2.0 ST Gene Expression Microarrays was performed pointing to a crucial role of Mc1r and Nr4a1 signaling in NDP-MSH-mediated neuroprotection. Electrophysiology studies demonstrating that the NDP-MSH-mediated restoration of action potential firing even under excitotoxic conditions is dependent on the presence of functional melanocortin-1 and nuclear orphan 4 receptors confirmed the gene expression data. To directly translate these observations, human neurons were generated from progenitor cells and treated with glutamate to induce excitotoxicity in the absence and presence of NDP-MSH showing that also in human neurons NDP-MSH mediates direct neuroprotection via signaling through MC1R and NR4A1. Additionally, MC1R and NR4A1 were quantified in human neuronal cells and brain tissue from healthy donors as well as MS patients revealing a markedly down-regulated MC1R expression in MS brain. Thus, the EMA-approved drug NDP-MSH, which is characterized by a high affinity to MC1R might represent a novel option for the treatment of inflammatory and degenerative diseases of the CNS.

Of note, all animal experiments and studies involving human material were approved by the responsible authorities as indicated in the methods. Experiments were conducted until statistical significance was reached and a simple randomization to the treatment groups (control versus NDP-MSH) was achieved by flipping a coin. In none of the experiments animals or samples were excluded from data analyses, which in some figures might have resulted in slightly increased error bars.
Mice

C57BL/6 (wild-type; purchased from Janvier-Labs, Cedex, France), DEREG (29), C57BL/6J/e (characterized by a point mutation in the Mc1r gene leading to a non-functional receptor, The Jackson Laboratory, Bar Harbor, MA), TCR\textsubscript{MOG} x IgH\textsubscript{MOG} (28), CD11c.DOG (30) and NGFI-B\textsubscript{–/–} mice (Nr4a1\textsubscript{–/–}) (37) were used at the age of 8-12 weeks and housed under specific pathogen-free conditions in micro-isolator cages. Mice were given chow and water \textit{ad libitum} and monitored for clinical signs daily. Consistent with animal welfare law, mice received a high-fat and high-protein diet beginning at a clinical score of 3. All animal experiments were performed with the approval of the State Review Board of North Rhine-Westphalia according to the German law for animal welfare, reference number 84-02.04.2013-A139.

Induction of EAE

EAE was induced by subcutaneous injection of 200 μg MOG peptide (MEVGWYRSPFSRVVHYLRNGK; Charité, Berlin, Germany) emulsified in complete Freund’s adjuvant (Sigma-Aldrich, Taufkirchen, Germany) containing 200 μg Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). Pertussis toxin (400 ng; Enzo Life Sciences, Lörrach, Germany) in 200 μL PBS was injected i.p. at the day of immunization and 2 days later. Disease severity was scored daily in a blinded fashion by two independent investigators using a scale from 0 to 10 (experimental autoimmune neuritis score) (54).

Pharmacological treatment of mice

α-MSH, KPV, KdPT or NDP-MSH (all purchased from Bachem, Bubendorf, Switzerland) were injected i.v. at a concentration of 5 μg in 100 μl PBS every 48 h when immunized mice reached a clinical score of ≥2. Control mice received an equal amount of PBS (PAA Laboratories, Pasching, Austria).
Depletion of Treg and CD11c⁺ DC

Depletion of Treg was achieved by i.p. injection of diphtheria toxin (DT, Sigma-Aldrich) at a concentration of 50 ng/g body weight into DEREG mice. CD11c⁺ DC were depleted by i.p. injection of DT at a concentration of 8 ng/g body weight into CD11c.DOG mice. DT was injected on a daily basis for 6 consecutive days starting 2 days before MOG immunization. Ablation of Treg or DC was verified before MOG injection as well as at days 8, 12, and 16 after immunization by multicolor flow cytometry.

Cell preparation and flow cytometry

Single-cell suspensions of lymph nodes, spleen and peripheral blood were prepared according to standard methods. For the isolation of mononuclear cells from the CNS (brain and spinal cord), mice were perfused with PBS through the left ventricle. Subsequently, tissues were homogenized through 100-µm cell strainers to obtain single-cell suspensions and Percoll gradient centrifugation was performed.

The expression of cell surface and intracellular markers was analyzed by multicolor flow cytometry on a Gallios flow cytometer (Beckman Coulter, Krefeld, Germany) using the Kaluza software. For flow cytometry, cells were stained in PBS using antibodies against CD4 (clone RM4-5), CD11b (clone M1/70), CD11c (clone N418), CD80 (clone 16-10A1), CD86 (clone GL1), MHC II (clone M5/114), PD-L1 (clone 10F.9G2; all purchased from Biolegend, San Diego, CA). Intracellular staining of CTLA-4 (clone UC10-4B9), Foxp3 (clone FJK-16s), Helios (clone 22F6), IDO (clone mIDO-48), IFN-γ (clone XMG1.2), IL-10 (clone JES5-16E3), IL-12 (clone C17.8), IL-17A (clone TC11-18H10.0), IL-22 (clone Poly5164), ROR-γt (clone REA278), Tbet (clone 4B10), TGF-β1 (clone TW7-16B4) and TNF-α (clone MP6-XT22; all purchased from Biolegend or Miltenyi Biotech, Bergisch-Gladbach, Germany) was performed after cell permeabilization using the Fix/Perm Buffer Set (Biolegend) according to the manufacturer’s instructions. Isotype-matched controls were included in each staining and apoptotic cells were identified using an annexin V apoptosis detection kit.
Proliferation and suppression assays

CD4+ T-cell subsets and DC were negatively enriched by magnetic cell separation using the appropriate kits (Miltenyi). T-cells were stained with 1 µM carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Frankfurt, Germany) and co-cultured with DC pulsed with 10 µg/mL MOG peptide (DC:T-cell ratio = 1:20) for 3–5 days at 37°C and 5% CO2. Subsequently, CFSE dilution, as a measure of cell proliferation, was quantified by flow cytometry. Dead cells were excluded using TO-PRO-3 iodide staining (Life Technologies).

Suppression assays were performed by labeling 1.5 x 10^5 CD4+CD25– effector T-cells from α-MSH- treated, NDP-MSH-treated mice or PBS-treated controls with 1 µmol/L CFSE and co-culturing them with CD4+CD25+ Treg for 4–5 days in a 1:1 or 2:1 ratio in the presence of anti-CD3 and anti-CD28 (clones 145-2C11 and 37.51; 0.5 µg/mL each antibody). The suppressive activity of Treg was assessed by the inhibition of effector T-cell proliferation in response to stimulation with mitogenic antibodies and quantified by CFSE dilution.

Hippocampal neuronal cell cultures and glutamate excitotoxicity assays

Neuronal cell cultures were obtained from wild-type, Nr4a1−/− or C57BL/6J e/e embryos (E18) following previously described protocols (55). Neuronal cultures were incubated at 37°C and 5% CO2 for 5-7 days and stimulated with 1 nmol/L NDP-MSH or an equal amount of PBS two times per day for the last 3 days of the culture. Analysis of excitotoxic neuronal cell damage was performed by treating neuronal cells for 6 hours in standard artificial cerebrospinal fluid (ACSF; 120 mmol/L NaCl; 2.5 mmol/L KCl; 1.25 mmol/L NaH2PO4; 22 mmol/L NaHCO3; 2 mmol/L MgSO4; 2 mmol/L CaCl2; 20 mmol/L dextrose; pH 7.35 adjusted by bubbling with a mixture of 95% O2 and 5% CO2) with 50 µM L-glutamate (Sigma-Aldrich) in the presence or absence of NDP-MSH. Subsequently, neurons were used for gene expression analyses or were fixed in 4% paraformaldehyde for immunofluorescence staining.
Human neuronal cell cultures and glutamate excitotoxicity assays

Human neuronal cells were generated from progenitor cells (ReNcell VM; Merck-Millipore, Temecula, CA) as described (56). After differentiation, neurons were pretreated with 1 nmol/L NDP-MSH for 3 days. The analysis of excitotoxic neuronal cell damage was performed as described above.

Histology and immunofluorescence staining

Mouse tissues (brain, spinal cord) were cryopreserved in NEG50 (Thermo Fisher Scientific, Waltham, MA), cut into 3-µm sections and immunofluorescence staining was performed using standard methods and the appropriate dilutions of primary antibodies against CD31 (clone ER-MP12, Thermo Fisher Scientific), glial fibrillary acid protein (GFAP; clone G-A-5, Sigma Aldrich), Iba1 (rabbit polyclonal, Abcam, Cambridge, UK), CD11c (clone N418, Biolegend), CNPase (Clone 11-5B, Sigma Aldrich), CD4 (clone RM4-5), RILPL2 (Proteintech, Chicago, IL), APP (rabbit polyclonal, Cell Signaling Technology, Frankfurt, Germany), IL-17A (clone eBio17B7), NeuN (clone A60; Merck-Millipore), Lama5 (kindly provided by Prof. Dr. L. Sorokin), Foxp3 (clone FJK-16s), Nr4a1 (Novus Biologicals, Cambridge, UK), Mc1r (Biozol, Eching, Germany), cleaved-caspase-3 (clone 9664; Cell Signaling Technology). Subsequently, slides were incubated with AlexaFluor (AF)-647-, AF594-, AF488- or AF421-coupled secondary antibodies (Life Technologies). Isotype controls were included in each staining and in some experiments nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

For hematoxylin and eosin (H&E) as well as luxol fast blue (LFB; Sigma-Aldrich) staining tissues were embedded in paraffin and cut into 3µm sections. After deparaffinization using standard protocols, H&E staining was performed using an autostainer (Tissue-Tek Prisma, Sakura Finetek, Torrance, CA). For LFB staining slides were incubated with LFB solution (Sigma-Aldrich) overnight, rinsed with distilled water and differentiated with 0.05 % lithium carbonate. Subsequently, slides were analyzed on an Olympus BX63 microscope using the cellSens software (Olympus, Münster, Germany).
Human autopsy and biopsy materials from patients with MS were obtained from the Netherlands Brain Bank (NBB), Amsterdam. The NBB provides its ethical authorization for "donation and collection of brain material for scientific research" (Ref. 2009/148; n = 3 subjects). Written informed consent was obtained from all patients and experiments were carried out according to the declaration of Helsinki. The lesions fulfilled the morphologic criteria of an inflammatory demyelinating process consistent with MS when stained with H&E, LFB, and periodic acid Schiff (PAS), myelin stain and Bielschowsky’s silver impregnation for axons. We classified lesions according to their demyelination status and included early and late active lesions. Staining was performed on 10-μm coronal sections.

**RNA isolation and quantitative real-time PCR (qPCR)**

RNA was extracted from snap-frozen tissues or purified cells using the innuPREP-RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA using random hexanucleotide primers and the RevertAid First strand cDNA Synthesis Kit (Thermo Fisher Scientific). qPCR was performed in a qTower 2.2 real-time PCR system (Analytik Jena) using the KAPA SYBR FAST Universal qPCR Master Mix (VWR International, Radnor, PA). All reported mRNA levels were normalized to β-actin or GAPDH and primer sequences are depicted in Supplementary Table 1. Relative mRNA expression was calculated according to the 2^{-ΔΔct} method (57).

**Bio-Plex assays**

Neuronal cells were isolated and stimulated as described. Subsequently, cell lysates were prepared using the Cell Lysis Buffer (Bio-Rad, Munich, Germany) according to the manufacturer’s instructions. Total CREB, p38 MAPK, phospho-CREB (Ser^{133}) and phospho-p38 MAPK (Thr^{180}/Tyr^{182}) levels were assessed by using Bio-Plex Pro™ Magnetic Cell Signaling Assays and a Bio-Plex Magpix™ multiplex reader (Bio-Rad) according to the manufacturer’s protocols. Phospho-proteins were quantified as percentage relative to the total amount of proteins. Cell lysate controls (Bio-Rad) were included in each assay.
Electrophysiology

Neuronal cells were isolated and stimulated as described. Electrophysiology measurements were conducted in the whole-cell current-clamp configuration of the patch-clamp technique. Recording pipettes were purchased from borosilicate glass (GT150TF-10, Clark Electromedical Instruments, Pangbourne, UK; typical resistance was 4–7 MΩ) and filled with an intracellular solution containing (in mmol/L): K-gluconate, 95; K₃-citrate, 20; NaCl, 10; HEPES, 10; MgCl₂, 1; CaCl₂, 0.5; BAPTA, 3; Mg-ATP, 3; and Na₃-GTP, 0.5. The internal solution was set to a pH of 7.25 with KOH and an osmolarity of 295 mOsm/kg. ACSF was used as extracellular solution containing (in mmol/L): NaCl, 120; KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 22; MgSO₄, 2; CaCl₂, 2; glucose, 20; pH 7.35 (adjusted by bubbling with a mixture of 95% O₂ and 5% CO₂) and osmolarity was set to 305 mOsm/kg. Membrane potentials were recorded using an EPC-10 amplifier, and digital analysis was done using Fitmaster (HEKA Elektronik, Lamprecht, Germany) and TRACE software.

Magnetic resonance imaging (MRI)

MRI was performed with a 9.4-T small animal MRI scanner (Biospec 94/20, gradient strength 700 mT/m) equipped with a helium-cooled Cryoprobe (BrukerBioSpin MRI GmbH, Ettlingen, Germany). Two anatomical T1-weighted 3D scans were acquired, one before and one 2 minutes after intravenous administration of 100 µmol/kg Magnevist into the tail vein, using a gradient echo sequence with TR/TE: 25/3 ms, FA: 15°. Geometry parameters were: FOV 15 × 15 mm, acquisition matrix of 160 × 160 × 96, resulting in an in plane resolution of (94 μm). Scan time per animal was 12 min 48 s. Scanning was performed under inhalation anesthesia using 1.5% isoflurane (1 L/min in O₂/compressed air, 20/80).

Injection of FITC-labeled dextran particles

Amounts of 25 mg/mL of 3-kDa dextran (Life Technologies) were diluted in PBS and injected i.v. into MOG-immunized mice (2.5 mg/mouse) at disease maximum 1 h before sacrifice. After perfusion spinal cord, brain, and kidney tissues were isolated and homogenized. Subsequently,
supernatants were used for fluorescence quantification on a TECAN Infinite M200PRO machine (TECAN, Männedorf, Switzerland) at 494 nm/521 nm. Fluorescence intensities in spinal cord and brain were normalized to kidney tissue.

**Generation of bone marrow chimeric mice.** Eight-week-old recipient mice were sublethally irradiated following standard procedures (58) using a Faxitron irradiator (Flaxitron Innovative Röntgentechnik, Much, Germany). Subsequently, mice were reconstituted with $7.5 \times 10^6$ bone marrow cells isolated from femurs and tibias of adult $Nr4a1^{-/-}$ or wild-type donors on a $Cd45.1$ or $Cd45.2$ background. Six to 12 weeks after bone marrow reconstitution, peripheral blood chimerism was assessed by flow cytometry.

**Gene expression profiling**

Gene expression profiling was performed on total RNA extracted from 5–10 mg of brain and spinal cord tissue from wild-type and C57BL/6J $e/e$ mice treated with PBS or NDP-MSH at disease maximum. Afterwards, total RNA preparations were analyzed for integrity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples showed high quality (mean RNA Integrity Numbers 9.3). RNA was further analysed by photometric Nanodrop measurement and quantified by fluorometric Qubit RNA assays (Life Technologies). Synthesis of biotin-labeled cDNA was performed according to the manufacturer’s protocols (WT Plus Reagent Kit; Affymetrix Inc., Santa Clara, CA). Briefly, 100 ng of total RNA were converted to cDNA. After amplification by *in vitro* transcription and 2nd cycle synthesis, cDNA was fragmented and biotin-labeled by terminal transferase. Finally, end-labeled cDNA was hybridized to Affymetrix Mouse Gene 2.0 ST Gene Expression Microarrays for 16 h at 45 °C, stained by streptavidin/phycoerythrin conjugate and scanned as described in the manufacturer’s instructions. Data analyses on Affymetrix CEL files were conducted using the GeneSpring GX software (Vers. 12.5; Agilent Technologies, Palo Alto, CA). Probes within each probeset were summarized by GeneSpring’s ExonRMA16 algorithm after quantile normalization of probe level signal intensities across all samples to reduce inter-array variability (59). Input data pre-processing was concluded by baseline transformation.
to the median of all samples. After grouping of biological replicates (duplicates) according to their respective experimental condition a given probeset had to be expressed above background (i.e. fluorescence signal of a probeset was detected within the 20th and 100th percentiles of the raw signal distribution of a given array) in both replicates in at least one of two, or both conditions to be further analysed in pairwise comparisons. Differential gene expression was statistically determined by moderated t-tests. The significance threshold was set to $P = 0.05$. Differentially expressed genes passing a fold-change cut-off $>1.5$ and a $P$ value of $<0.05$ in all replicates of one experimental group were further characterized using the KOBAS 2.0 software (http://kobas.cbi.pku.edu.cn) and known as well as predicted interactions of proteins encoded by the differentially expressed genes were calculated using the STRING 10 software (http://string-db.org). The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE73587.

Statistics

All values are expressed as means ± SEM. Statistically significant differences were assessed by paired sign $t$-test (Fig. 3G; same individuals tested), Wilcoxon rank-sum test (Figs. 1A, 2A, 2G, 2H, 3A, 3D, 4A, 4D, 5D, 5F, 5H, 6F, 7C, S1, S2C, S3C, S3D, S5B, S5C, S6A, S9D, S10B; two independent groups - one treated one control, normal distribution not required), Student’s $t$-test (Figs. 1C, 1D, 1F, 2C, 3B, 3C, 6C, S2A, S3A, S5A, S7A, S7B, S7C, S7D, S10C; comparing two data sets following a normal distribution) or One-Way ANOVA test (Figs. 5A, 5B, 5C, 7A, 7B, S9A, S9C; comparing more than two groups). The $\alpha$ level was set at $<0.05$ in all cases and SigmaPlot 12 or GraphPad Prism 6 were used to analyze, plot, and illustrate data. Statistical analyses were done in collaboration with Robert Kwiecien, Institute of Biostatistics and Clinical Research, University of Münster, Germany.
LIST OF SUPPLEMENTARY MATERIALS

Supplementary materials include 1 table as well as 11 figures and can be found online:

Table S1: Sequences of primers used to amplify mouse and human genes.

Fig. S1: \( \alpha \)-MSH down-regulates the expression of markers associated with axonal damage.

Fig. S2: \( \alpha \)-MSH Inhibits EAE development by reducing the activity, antigen-specific proliferation and cytokine secretion in pathogenic Th1 and Th17 cells.

Fig. S3: \( \alpha \)-MSH expands immunosuppressive Treg in the regional lymph nodes from MOG-immunized mice.

Fig. S4: Signaling through a functional Mc1r is essential for the \( \alpha \)-MSH-mediated prevention of EAE.

Fig. S5: NDP-MSH generates tolerogenic DC with a reduced capacity to expand CD4\(^+\) effector T cells.

Fig. S6: \( \alpha \)-MSH efficiently inhibits the progression of ongoing EAE.

Fig. S7: Depletion of DC or Treg in DT-treated CD11c.DOG or DEREGR mice, respectively.

Fig. S8: Functional protein association networks and pathway classification of gene expression data from the CNS of MOG-immunized mice treated with PBS or NDP-MSH at disease maximum.

Fig. S9: Under inflammatory or excitotoxic conditions Mc1r ligation up-regulates \( Nr4a1 \) and allows AP generation.

Fig. S10: Disease progression and numbers of Th17, Th1 as well as Treg cells in the CNS from bone marrow chimeric mice.

Fig. S11: \( Nr4a1 \) expression in non-hematopoietic cells of the brain.
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Author contributions. N.S. performed most of the experiments; A.H. isolated murine hippocampal neurons and conducted electrophysiology experiments; N.Sch. helped with cell sorting; T.S., A.L. and L.K. generated and provided mouse mutants; C.F. and L.W. performed MRI analyses; R.D. and K.K. conducted the gene expression studies; T.A.L. and H.W. provided advice and contributed expertise; K.L. conceived the study, analyzed data, and together with S.G.M. wrote the manuscript.

Competing interests. The authors declare that they have no financial interests or conflicts of interest.
Figure 1

A

Clinical score

Body weight (%)

Days after immunization

Days after immunization

B

H&E

LPB + Eosin

Control

α-MSH

C

SSC

CD4

IL-17

ROR-γt

T-bet

IFN-γ

α-MSH

Control

% Pos cells in total CD4

D

SSC

CD4

Fosp3

Helios

TGF-β

GITR

α-MSH

Control

% Pos cells in total CD4

E

CD4+ Fosp3+ DAPI

Control

α-MSH

F

SSC

MHC-II

CD80

CD90

IFN-γ

PD-L1

α-MSH

Control

% Pos cells in total DC

G

Clinical score

Body weight (%)

Days after immunization

Days after immunization

H

C57BL/6, + α-MSH

C57BL/6, + α-MSH

IL-17

ROR-γt

Fosp3

Helios

α-MSH

α-MSH

8.9

10.8

5.4

4.9

31
Fig. 1. α-MSH prevents EAE by inducing functional Treg. (A) Wild-type mice \((n = 22\) per group) were immunized with MOG peptide and treated with α-MSH or PBS at indicated time points (arrows). Mean clinical scores (left) and loss of body weight (right) are shown. (B) Representative H&E and luxol fast blue (LFB) staining in lumbar spinal cord at disease maximum. Scale bars represent 50 μm, infiltration of mononuclear cells and demyelination is indicated by arrows. (C) Flow cytometry of effector T-cells in the CNS at disease maximum. Representative dot plots (left) and statistics from \(n = 8\) mice per group (right) are shown, cells are gated for CD4. (D,E) Flow cytometry (D) and immunofluorescence staining (E) of Treg in the CNS at disease maximum. Representative dot plots of Helios+ or Helios− Foxp3+ Treg (left, gating strategy is depicted) and statistics from \(n = 8\) mice (right, gated for CD4) are shown. (D). Immunofluorescence staining of brain tissue from PBS- and α-MSH-treated mice at disease maximum using antibodies against CD4 (red) and Foxp3 (green). Scale bars represent 25 μm (left) or 75 μm (right), nuclei are counterstained with DAPI (left) (E). (F) Flow cytometry of DC from α-MSH- and PBS-injected mice at disease maximum. Representative dot plots (left) and statistics from \(n = 8\) mice (right) are shown. Cells are gated for MHC II+CD19−F4/80−. (G) C57BL/6J/e mice \((n = 15)\) were immunized with MOG peptide and treated with α-MSH at indicated time points (arrows). Mean clinical scores (left) and loss of body weight (right) are shown. (H) Flow cytometry of Th17 and Treg cells in the CNS at disease maximum. Representative dot plots are shown, cells are gated for CD4. All plots are presented as mean ± SEM; \(P < 0.05\) vs. PBS-treated controls.
Fig. 2. α-MSH and NDP-MSH efficiently inhibit the progression of ongoing EAE. (A) Wild-type mice (n = 22 in each group) were immunized with MOG peptide and treated with NDP-MSH or PBS after onset of disease in 48-h intervals (arrows). Mean clinical scores (left) and loss of body weight (right) are shown. (B,C) Flow cytometry of effector T-cells and Treg in the CNS at disease maximum. Representative dot plots (B) and statistics from n = 9 mice (C) are shown, cells are gated for CD4. (D) Representative H&E and luxol fast blue (LFB) staining in lumbar spinal cord at disease maximum. Scale bars represent 50 μm and infiltration of mononuclear cells or demyelination is indicated by arrows. (E) Representative immunofluorescence staining of spinal cord tissue from mice treated with NDP-MSH or PBS at disease maximum using antibodies against APP, RILPL2 and the neuronal marker NeuN. Scale bars represent 20 μm. (F) MRI of brains from NDP-MSH-treated mice and controls at disease maximum. Two representative images of a multiplanar reconstruction per group are shown (out of n ≥ 4 mice that have been analyzed per group). Signal enhancement in the brain due to contrast agent accumulation is marked by arrows. (G) Immunofluorescence staining of spinal cord tissue from mice treated with NDP-MSH or PBS at disease maximum using antibodies against CD4 (red), IL-17 (green) and Lama5 (gray). Representative images from 2 mice (top) as well as the statistical evaluation from 30 visible fields out of n = 6 mice per group are shown. Scale bars represent 20 μm, reduced Lama5 expression in the basement membrane is indicated by arrows. (H) Relative fluorescence intensity in the spinal cord as well as different brain regions of MOG-immunized mice treated with NDP-MSH or PBS and injected with FITC-labeled dextran particles (n = 8 spinal cord or n = 5 brain regions). All plots are shown as mean ± SEM; P < 0.05 vs. PBS-treated controls.
Fig. 3. Therapeutic treatment with NDP-MSH attenuates disease progression in Devic mice. (A) After disease onset, Devic mice (n = 7–10) were injected with NDP-MSH or PBS in 48-h intervals starting at the indicated time point (arrow). Mean clinical scores (left) and body weight (right) are shown. PBS-treated mice had to be euthanized at d50 (†). (B,C) Flow cytometry of total CD4+ T-cells in the lumbar spinal cord (B) and Th1, Th17 and Treg cells in regional lymph nodes (C) at disease maximum (d50 after birth). Representative histograms (B, left) and statistics from n = 6 mice (B, right and C) are shown. Cells are gated for CD4 (C). (D) H&E, LFB, RILPL2, APP and NeuN staining in lumbar spinal cord from NDP-MSH- and PBS-treated Devic mice at d50 after birth. Scale bars represent 50 µm (H&E, LFB) or 20 µm (RILPL2, APP, NeuN). One representative image (left) and statistics from n = 6 mice (right) are shown. Infiltration of mononuclear cells (H&E) and areas of demyelination (LFB) are indicated by arrows. (E) Immunofluorescence staining of spinal cord tissue using antibodies against CD4 (red), IL-17; (green) and Lama5 (gray). One representative image is shown, scale bars represent 20 µm and reduced Lama5 expression is indicated by arrows. (F) Treatment with NDP-MSH from d36 to d64 prevented Devic mice from relapse for >8 weeks after cessation of therapy. Clinical scores from n = 8 mice are depicted (individual mice are marked by different symbols). (G) Flow cytometry of CD4+ T-cells in the lumbar spinal cord from NDP-MSH-treated Devic mice at d124 and d194 after birth. Representative histograms (left) and statistics from n = 7 mice (right) are shown. Data are presented as mean ± SEM; *P < 0.05 vs. Devic, d194. (H) H&E, LFB, and immunofluorescence staining using antibodies against CD4 (red), IL-17 (green) and Lama5 (gray) in lumbar spinal cord from NDP-MSH- and PBS-treated Devic mice at d124 and d194 after birth. One representative image is shown. Scale bars represent 50 µm (H&E, LFB) or 20 µm (immunofluorescence). Infiltration of mononuclear cells (H&E), areas of demyelination (LFB) and reduced Lama5 expression in the basement membrane are indicated by arrows. All plots are shown as mean ± SEM; P < 0.05 vs. PBS-treated controls.
Fig. 4. Depletion of Treg or DC does not abrogate the beneficial effect of NDP-MSH. (A) DEREG mice ($n = 8$) were injected with diphtheria toxin (DT), immunized with MOG peptide and treated with NDP-MSH at indicated time points (arrows). Mean clinical scores (left) and loss of body weight (right) are shown. (B,C) Flow cytometry of Th1 and Th17 cells in the CNS at disease maximum. Representative dot plots (B) and statistics from $n = 8$ mice per group (C) are shown. Cells are gated for CD4. (D) After depletion of DC by injecting DT, CD11c.DOG mice ($n = 8$) were immunized with MOG peptide and treated with NDP-MSH at indicated time points (arrows). Clinical score (left) and loss of body weight (right) are shown. (E,F) Flow cytometry of Th1, Th17 and Treg cells in the CNS at disease maximum. Representative dot plots (E) and statistics from $n = 8$ mice or $n = 3$ mice (CTLA-4, GITR, IL-10 and TGF-β staining) (F) are depicted. Cells are gated for CD4. All plots are shown as mean ± SEM; $P < 0.05$ vs. PBS-treated controls.
Fig. 5. NDP-MSH prevents neuronal damage and preserves AP generation under excitotoxic conditions by up-regulating Nr4a1. (A) Immunofluorescence staining of mouse neurons after stimulation with glutamate (Glu) in the absence or presence of NDP-MSH using antibodies against Nr4a1, Mc1r, cleaved caspase 3 (Casp3) and NeuN. One representative (top) and statistics from $n = 3$ different neuronal cell cultures (bottom) are shown. Scale bars represent 20 µm and in some experiments, nuclei are counterstained with DAPI. (B) qPCR to quantify the expression of Casp8 and Casp3 in isolated hippocampal neurons after stimulation with Glu in the absence or presence of NDP-MSH. mRNA levels from $n = 4$ different neuronal cell cultures are shown. (C) Relative levels of phosphorylated CREB (Phospho-CREB) and Phospho-p38 MAPK in cell lysates of NDP-MSH-treated wild-type, C57BL/6J $^{+/+}$ and $Nr4a1^{-/-}$ neurons in the presence and absence of Glu ($n = 3$ different neuronal cell cultures). (D) Membrane potentials and numbers of action potentials (AP) in PBS- ($n = 5$, black) or NDP-MSH-treated ($n = 6$, gray) hippocampal neurons isolated from wild-type mice in the absence or presence of Glu. (E) Firing behavior of PBS- and NDP-MSH-treated wild-type neurons in the presence or absence of Glu provoked by depolarizing current steps (stimulation protocol). (F,G) Membrane potentials, numbers of AP (F) and firing behavior (G) of PBS- ($n = 5$, black) and NDP-MSH-treated ($n = 3$, gray) C57BL/6J $^{+/+}$ hippocampal neurons in the presence or absence of Glu. (H,I) Membrane potentials, numbers of AP (H) and firing behavior (I) of PBS- ($n = 6$, black) and NDP-MSH-treated ($n = 5$, gray) $Nr4a1^{-/-}$ hippocampal neurons in the presence and absence of Glu. Scale bars represent 500 ms and 50 mV (E,G,I) and all plots are shown as mean ± SEM; *$P < 0.05$ vs. Glu + PBS treatment.
Figure 6

A

Clinical score

Days after immunization

B

PBS

NDP-MSH

IL-17

ROR-α

Foxp3

Helios

C

% Pos. cells

in total CD4

IL-17

ROR-α

IL-22

IFN-γ

Foxp3

N4a1

N4a1

D

H&E

LFB - Eosin

RILPL2 - NeuN

APP - NeuN

N4a1

N4a1

E

CD4 - IL-17 - Lamin5

N4a1

N4a1

F

Clinical score

Days after immunization

G

H&E

LFB - Eosin

CD4 - IL-17 - Lamin5

PBS

NDP-MSH

PBS

NDP-MSH

41
Fig. 6. The neuroprotective effect of NDP-MSH is dependent on Nr4a1. (A) Development of EAE in Nr4a1<sup>−/−</sup> mice treated with NDP-MSH or PBS at indicated time points (arrows). Mean clinical scores from n = 8 mice per group are shown. (B,C) Flow cytometry of Th1, Th17, and Treg cells in the CNS at disease maximum. Representative dot plots (B) and statistics from n = 4 mice (C) are shown. Cells are gated for CD4. (D) Representative H&E, LFB staining (left) and immunofluorescence staining (right) using antibodies to RILPL2, APP and NeuN in lumbar spinal cord at disease maximum. Scale bars represent 50 µm (H&E, LFB) or 20 µm (immunofluorescence). One representative image (left) and statistics from n = 4 mice (right) are shown. Areas of demyelination (LFB) are indicated by arrows. (E) Immunofluorescence staining of spinal cord tissue using antibodies against CD4 (red), IL-17 (green) and Lama5 (gray). One representative image is shown. Scale bars represent 20 µm. Reduced Lama5 expression in the endothelial basement membrane is indicated by arrows. (F) Development of EAE in bone marrow chimeric mice treated with NDP-MSH or PBS at indicated time points (arrows). Mean clinical scores from n ≥ 6 mice per group are shown. (G,H) Representative H&E, LFB (left) and immunofluorescence staining (right) using antibodies to CD4 (red), IL-17 (green) and Lama5 (gray) in Nr4a1<sup>−/−</sup> recipients of wild-type (Cd45.1) bone marrow (G) and wild-type (Cd45.1) recipients of Nr4a1<sup>−/−</sup> bone marrow (H). Scale bars represent 50 µm (H&E, LFB) or 20 µm (immunofluorescence). Infiltration of mononuclear cells (H&E), areas of demyelination (LFB) and reduced Lama5 expression is indicated by arrows. All plots are shown as mean ± SEM; P < 0.05 vs. PBS-treated controls.
Figure 7

A

mRNA expression

[p-fold rel. to ACTB]

MC1R  NR4A1

Control  Glu  NDP-MSH + Glu

mRNA expression

[p-fold rel. to ACTB]

CASP8  CASP3

B

Control  Glu  NDP-MSH + Glu

NR4A1 • MC1R • DAPI

CASP3 • NEUN

% MC1R • NR4A1

% CASP3 • NEUN

C

NR4A1 • MC1R • NEUN

Healthy human brain  MS

% MC1R • NR4A1

% MC1R • NR4A1

Healthy human brain  MS
Fig. 7. NDP-MSH protects human neuronal cells from glutamate-induced apoptosis. 

(A) mRNA expression of MC1R, NR4A1, CASP8, and CASP3 in human neuronal cells differentiated from progenitor cells and stimulated with glutamate (Glu) in the absence or presence of NDP-MSH (n ≥ 4 different neuronal cell cultures). *P < 0.05 vs. Glu + PBS treatment. 

(B) Immunofluorescence staining of human neuronal cells after stimulation with Glu in the absence or presence of NDP-MSH using antibodies against NR4A1, MC1R, CASP3 and the neuronal cell marker NEUN. One representative image (left) and statistics from n = 4 different neuronal cell cultures (right) are shown. In some experiments nuclei are counterstained with DAPI. Scale bars represent 20 µm; *P < 0.05 vs. Glu + PBS treatment. 

(C) Immunofluorescence staining of brain tissue from human donors or individuals with MS using antibodies against NR4A1, MC1R, and NEUN. Two representative images (left) and statistics from n = 3 different subjects (right) are shown. Scale bars represent 20 µm; *P < 0.05 vs. healthy human brain. All plots are shown as mean ± SEM.
Fig. 8. Schematic illustration of NDP-MSH-mediated anti-inflammatory and neuroprotective effects. (A) EAE is characterized by the infiltration of peripherally generated autoreactive Th1 and Th17 cells into the CNS resulting in tissue damage to oligodendrocytes and demyelination. Th1 and Th17 activity is controlled by Treg. (B) NDP-MSH expands functional Treg that efficiently suppress pathogenic Th1 and Th17 cells in ongoing EAE, improves BBB integrity by inducing a continuous Lama5 expression and, additionally, mediates a strong and long-lasting neuroprotective effect, including the restoration of action potential firing via signaling through Mc1r and Nr4a1.