

Prophylactic and Therapeutic Modulation of the OGF-OGFr Axis Ameliorates Angiogenesis-associated Pathology in Experimental Autoimmune Encephalomyelitis

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease associated with inflammation and neurodegeneration of the central nervous system. MS pathogenesis includes angiogenesis, the formation of new blood vessels from existing vasculature. Angiogenesis facilitates the migration of inflammatory immune cells across the blood-brain barrier in MS. The opioid growth factor (OGF)-opioid growth factor receptor (OGFr) axis is a potential target for limiting angiogenesis in MS. OGF is a ubiquitous endogenous opioid with inhibitory growth properties when bound to OGFr. Low dosages of naltrexone (LDN), an opioid receptor antagonist, transiently block OGFr and lead to upregulated OGF production when naltrexone is unbound. To investigate the role of the OGF-OGFr axis in angiogenesis in a preclinical model of MS, C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) to establish chronic progressive experimental autoimmune encephalomyelitis (EAE). Mice were treated with intraperitoneal OGF (10 mg/kg) or LDN (0.1 mg/kg) prophylactically for 10 days or therapeutically for 10 days or 3 weeks. Prophylactically treated EAE + OGF or EAE + LDN mice had markedly reduced spinal cord inflammation (OGF: $p=0.03$; LDN: $p<0.01$) and white matter blood vessel density (OGF: $p<0.0001$; LDN: $p<0.0001$) compared to vehicle treated EAE mice. Therapeutic EAE + OGF or EAE + LDN mice had improved clinical behavior and reduced white matter blood vessel density (OGF: $p=0.05$; LDN: $p<0.05$) with an insignificant decrease in spinal cord inflammation (OGF: $p=0.15$; LDN: $p=0.53$) after 3 weeks of treatment. This study is the first to demonstrate a role for LDN and the OGF-OGFr axis in reducing angiogenesis in EAE mice. Most current MS therapies have significant side-effects and high costs, while LDN has been shown to be safe and inexpensive. This study provides preclinical evidence for LDN as a promising therapy for limiting MS pathophysiology.

Keywords: Multiple sclerosis, Experimental autoimmune encephalomyelitis, Angiogenesis, Neuroinflammation, Opioid growth factor, Opioid growth factor receptor, Low-dose naltrexone

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that impacts approximately 1 million individuals in the US and 2.9 million individuals worldwide [1]. MS manifestation and progression involves inflammation, demyelination, and axonal damage leading to sensorimotor, psychiatric, and cognitive symptoms. Active MS lesions result from T- and B-cell migration to the CNS, wherein they facilitate inflammation, gliosis, and demyelination. These peripheral immune cells migrate across the blood-brain barrier (BBB), which becomes more expansive and leakier in

MS through angiogenesis, the process of new blood vessel formation from existing vasculature [2].

During angiogenesis, endothelial cells proliferate and migrate, increasing vascular surface area and paracellular permeability through the loss of intercellular junctions [3]. These processes facilitate immune cell infiltration of the CNS, and through the release of pro-angiogenic signaling molecules like vascular endothelial growth factor (VEGF), infiltrative immune cells and native CNS cells like reactive astrocytes and neurons perpetuate endothelial cell proliferation [2,4]. Some reports indicate that angiogenesis might be beneficial in later MS through the

recruitment of remyelinating elements; however, the concept that imbalanced pro- and anti-angiogenic signaling is harmful throughout most of the disease persists [5,6]. Numerous approved MS therapies target immune cell proliferation and cytokine production, but only one (natalizumab) targets immune cell entry to the CNS [7]. Natalizumab hinders all T-cell tethering to the BBB, potentially leading to often-fatal progressive multifocal leukoencephalopathy [7]. Therefore, there is a need for therapies that limit immune cell infiltration without completely depriving the CNS of T-cell-mediated immunity.

Angiogenesis underlies pathogenesis in experimental autoimmune encephalomyelitis (EAE), the foremost preclinical model of MS. BBB dysfunction and increased vessel density have been observed in the CNS as early as 4 days post-induction (dpi) in EAE mice, approximately one week before EAE symptom onset [8]. Less is known about the extent of angiogenesis in later EAE; however, studies report increased vessel density and VEGF expression as late as 19 dpi and 14 dpi, respectively [8,9]. Previous EAE studies have demonstrated that angiogenesis is a viable therapeutic target. Pharmacologic blockade of VEGF with bevacizumab, an anti-VEGF antibody, or sexamanib, a VEGF receptor inhibitor, reduced blood vessel density, immune cell infiltration, and demyelination in EAE mice [10,11]. However, safety concerns over bevacizumab and sexamanib, both of which carry significant risks of cardiopulmonary complications, necessitate further research on potential anti-angiogenic treatments that are safe and equally effective [12,13].

The current study was initiated to explore potential mitigating factors of angiogenesis in EAE. Blockade of the opioid growth factor (OGF)- opioid growth factor receptor (OGFr) axis may be a mechanism for limiting angiogenesis. OGF is an endogenous opioid that has inhibitory growth properties when bound to OGFr [14]. Administration of exogenous OGF inhibited angiogenesis, while naltrexone (NTX), a general opioid receptor antagonist, increased angiogenesis in a chick chorioallantoic membrane model [15]. NTX also increased angiogenesis in cutaneous wounds in a rat model of diabetes [16]. While normal doses of NTX are associated with continuous OGFr blockade, intermittent blockade of OGFr with low doses of NTX (LDN) promotes the upregulation of OGF and subsequently restores normal levels of OGF/OGFr binding once LDN is unbound [17]. There is a paucity of research on opioid systems in angiogenesis in MS and EAE. Studies on cancer have indicated that kappa opioid receptor (KOR) agonists are anti-angiogenic in tumor environments, and it is reasonable to suspect that these drugs may be beneficial in MS [18]. However, repeated use of KOR agonists leads to analgesic and potential angiogenic tolerance [19,20]. This makes naltrexone, an opioid receptor antagonist, an attractive potential therapy for MS-related angiogenesis.

To study the potential effects of modulating the OGF-OGFr axis on angiogenesis in EAE, we induced Ch-EAE in

female C57BL/6 mice and treated mice with OGF or LDN on a prophylactic (beginning 0 dpi) or therapeutic (beginning at disease onset, 11 dpi) timeline. The goal of the therapeutic paradigm was to determine if OGF or LDN treatment could rescue clinical disease behavior and sustain anti-angiogenic activity and reduce immune cell infiltration after 3 weeks of treatment. This paradigm is clinically relevant, as most patients have established disease (multiple lesions disseminated in space and time) at the time of diagnosis and treatment. The goal of the prophylactic paradigm was to determine if OGF or LDN treatment for 10 days could prevent angiogenesis and immune cell infiltration that precede symptom onset. The translational benefit of prophylactic treatment is that, in combination with earlier and less invasive biomarkers for pre-MS biological changes, MS severity could be limited prior to established disease onset.

Materials and Methods

Animals

Six- to seven-week-old female C57BL/6J mice (stock #000664) were purchased from the Jackson Laboratory, Bar Harbor, ME and acclimated for at least one week before EAE induction. Animals were housed 5 per cage in rooms that were maintained at $21 \pm 0.5^\circ\text{C}$ with a relative humidity of $50 \pm 10\%$ and a 12 h light-dark cycle with no twilight. Pellet food and sterile water were available *ad libitum*, and mice were provided with soft gel food and water *ad libitum* as needed. All experiments were conducted in accordance with Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee approval.

EAE induction and treatment

Mice were randomly selected for immunization ($n=50$) or to serve as nonimmunized controls ($n=15$). Chronic EAE was induced with two 0.1 mL subcutaneous injections of an emulsion of 400 μg myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) dissolved in complete Freund's adjuvant (#EK-2110, Hooke Laboratories). Two injections were placed in the midline, one each along the upper and lower back. Following MOG₃₅₋₅₅ injection, mice received 0.1 mL intraperitoneal (ip) injections of 110 ng pertussis toxin (PTX) in sterile phosphate-buffered saline (PBS) on days 0 and 1 post-induction to facilitate temporary blood-brain barrier permeabilization. Non-EAE control mice were injected with equal volumes of sterile phosphate-buffered saline (PBS). Mice were lightly anesthetized with 3% isoflurane (Vedco, Inc., St. Joseph, MO) for subcutaneous injections, but not for ip injections.

Mice immunized with MOG₃₅₋₅₅ were randomly assigned to treatment groups either on day 0 post-induction (dpi) for the prophylactic treatment cohort, or at the time of disease onset (11 dpi) for the therapeutic treatment cohorts. Mice in the prophylactic cohort received daily 0.1 mL ip injections of 10 mg/kg OGF ($n=7$), 0.1 mg/kg naltrexone (low dose naltrexone;

LDN) (n=6), or sterile PBS (n=6). Mice in the therapeutic cohorts received daily 0.1 mL ip injections of 10 mg/kg OGF (n=12), 0.1 mg/kg LDN (n=7), or sterile PBS (n=11). Non-EAE control mice were injected with equal volumes of PBS daily. Mice were treated prophylactically for 10 days or therapeutically for 10 days or 3 weeks. Injections were administered at approximately the same time (1200-1400) daily.

Clinical behavior

Beginning at 9 dpi, mice were observed daily for behavior indicating disease progression. Clinical behavior was evaluated on a scale of 0-10, with 0 representing no disease behavior and 10 representing moribundity or death due to EAE [21,22]. Clinical behavior scores were the summation of tail tone, gait, righting reflex, and individual limb function measurements that were observed for each mouse daily. Tail tone was scored as normal (0), distal tail tone loss (0.5), or complete loss of tone/flaccid (1). Gait was scored as normal (0), slight abnormality/limping gait (1), moderate abnormality (1.5), or severe abnormality (2). Righting reflex was scored based on the time it took mice to right themselves after being placed supine; scores were normal (0), slow (0.5), or absent (1). Limb function was scored for each limb as mice were placed on a metal grid and inverted for 20 seconds or based on the ability to move the limb at higher scores. A limb was determined weak (0.5) if the mouse had trouble grasping the grid but was able to support the weight of the mouse during ambulation. If a limb had limited movement during ambulation and was unable to support the weight of the mouse, it was near paralysis (1). A near-paralyzed limb is typically abducted to the sides of the mouse as the hindfeet "paddle" along the ground. A limb was deemed paralyzed (1.5) if it was incapable of any movement and body weight support. A paralyzed limb is typically cachexic and is dragged behind the mouse during ambulation. An animal with four-limb paralysis would also have absent tail tone, gait, and righting reflex, receiving a score of 10. Since an animal with this score cannot ambulate, this score was a humane endpoint of the study. Disease onset was determined as the second consecutive day when mice displayed a clinical score of 0.5 or greater. Animals were scored daily by an investigator masked to treatment groups.

Histopathology

At 9 dpi (prophylactic) or 21 or 34 dpi (therapeutic), deeply anesthetized mice were euthanized with intracardial perfusion of PBS followed by 4% paraformaldehyde (PFA), and intact vertebral columns were collected. Vertebral columns were post-fixed in 4% PFA overnight and dissected at the lumbar enlargement; rostral segments were frozen for immunofluorescence and caudal segments were paraffin-embedded for histological staining. Caudal spinal cord segments were dehydrated in increasing concentrations of alcohol and xylene, followed by immersion in melted paraffin wax. Paraffin-embedded sections (6 μ m) of the lumbar

region were stained with hematoxylin & eosin (H&E) for measurement of infiltrative immune cells. Using H&E-stained sections, white matter inflammation was scored 0-4 based on a previously described method: (0) no inflammation, (1) infiltrative cells detected only in the perivascular regions and meninges, (2) mild cellular infiltration in the white matter, (3) moderate cellular infiltration in the white matter, (4) severe cellular infiltration in the entirety of the white matter [23]. Histopathology scoring was performed by an investigator masked to treatment groups.

Immunohistochemistry

Rostral spinal cord segments were immersed sequentially in 15% sucrose and then 30% sucrose for cryoprotection before being snap-frozen in optimal cutting temperature (OCT) with dry ice-cooled isopentane. Frozen sections (10 μ m) of the lumbar region were probed with pre-conjugated *Lycopersicon esculentum* (tomato) lectin (1:100, #DL-1178-1, Vector Laboratories, Newark, CA) for blood vessel identification or co-labeled with anti-OGFr antibody (1:500, #A304-542A, Thermo Fisher, Waltham, MA; secondary antibody: Alexa Fluor 568 1:1000, #A11011, Thermo Fisher, Waltham, MA) and lectin for receptor localization on blood vessels. Blood vessel density was measured using % area of lectin staining in two 100 μ m² regions of interest in the grey matter and white matter for each animal using ImageJ (Fiji) [24]. OGFr localization on blood vessels was confirmed visually in two sections per mouse from each treatment cohort. Controls for immunostaining specificity were as follows: OGFr: primary antibody omitted (tissues incubated with secondary antibody only); lectin: lectin omitted (tissues incubated in blocking buffer only).

Statistical analysis

Data were analyzed using GraphPad Prism version 10.2 for MacOS (Boston, MA). For clinical behavior score analyses, a mixed-effects analysis of variance with Dunnett's multiple comparisons was performed. Spinal cord inflammation score data were analyzed with Kruskal-Wallis tests with Dunn's multiple comparisons correction. Blood vessel density data were analyzed using one-way ANOVAs with Tukey's multiple comparisons correction. For all tests, statistical significance was determined at $P < 0.05$.

Results

General observations

No normal (non-EAE) mouse developed neurological symptoms or died over the course of the therapeutic treatment experimental period (0-34 dpi). Over the course of the prophylactic treatment experimental period (0-9 dpi), none of the normal or EAE mice developed neurological symptoms or died (animals were euthanized prior to predicted symptom onset). One mouse in the prophylactic experiment

died on the day of induction, and one immunized mouse in the therapeutic experiment never developed EAE and was therefore excluded from analysis. One therapeutic treatment cohort (non-EAE n=5, EAE + PBS n=3, EAE + OGF n=5) was euthanized on 21 dpi due to humane endpoints being reached for 2 of the 5 PBS-treated mice on days 18 and 20 dpi. This therapeutic cohort was included in clinical behavior score analyses but not spinal cord pathology investigations due to the early experiment termination and loss of statistical power for between-groups comparisons at the 21 dpi timepoint. The other therapeutic cohort (non-EAE n=5, EAE + PBS n=4, EAE + OGF n=7, EAE + LDN n=7) was euthanized on 34 dpi after a completed treatment timeline; one EAE + PBS mouse was euthanized early (31 dpi) due to disease severity.

Therapeutic treatment with OGF or LDN ameliorates clinical behavior

Clinical behavior was scored from 0-10 by an investigator masked to treatment groups. MOG immunization led to first appearances of EAE clinical behavior on 10 dpi in the therapeutic treatment timeline; mean ($\pm$ SEM) score for all immunized mice was 0.4 ± 0.1 (Figure 1). At disease onset (11 dpi), the mean score for all immunized mice was 1.3 ± 0.3 . Disease onset was defined as the second consecutive day that mice had clinical scores >0 , and at this time mice were randomly assigned to receive daily OGF, LDN, or PBS. Clinical scores were comparable between all EAE groups until 21 dpi, when EAE + OGF mice had significantly lower scores (7.3 ± 0.4) compared to EAE + PBS mice (8.7 ± 0.3 ; $p<0.01$). From 26-29 dpi and 31-34 dpi (end of experiment), OGF treated mice had significantly lower scores than PBS treated mice. On 27 dpi and 31-34 dpi, LDN treated mice had significantly lower scores than PBS treated mice. Behavior scores were comparable for mice receiving the same treatments between the 2 therapeutic treatment cohorts, so scores were combined for between-group analyses.

Prophylactic treatment with OGF or LDN rescues spinal cord inflammation

Spinal cord inflammation was measured in H&E stained lumbar spinal cords from prophylactic and therapeutic mice. Inflammation was scored visually in the entirety of the white matter on a scale of 0-4 by an investigator masked to experimental conditions. As shown in representative images of the ventral spinal cord in Figures 2B-2C (area outlined in Figure 2A), in both treatment timelines, normal mice had no spinal cord inflammation. In the prophylactic cohort, EAE + LDN mice also had no inflammation and only one EAE + OGF mouse had mild spinal cord inflammation at 10 dpi. Normal, EAE + OGF, and EAE + LDN mice had significantly reduced inflammation scores compared to EAE + PBS mice (normal: $p<0.01$, EAE + OGF: $p<0.05$, EAE + LDN: $p<0.01$) (Figure 2D). In the therapeutic cohort, all EAE mice had some degree of spinal cord inflammation at 34 dpi. Normal mice had a significantly reduced mean inflammation score compared to EAE + PBS mice ($p<0.001$) (Figure 2E). EAE + OGF and EAE + LDN mice had reduced inflammation scores compared to EAE + PBS mice, but this difference was not significant. EAE + LDN mice had a significantly greater mean inflammation score than normal mice.

OGFr is present on spinal cord blood vessels

Lumbar spinal cord sections from mice in the prophylactic cohort were stained with anti-OGFr and lectin to determine the presence of OGFr on endothelial cells/blood vessels. Colocalization of OGFr on endothelial cells was observed in the spinal cords of non-EAE mice and EAE mice treated with OGF, LDN, or PBS (Figure 3; representative images from region outlined in Figure 2A). Since we did not anticipate differences in the degree of OGFr colocalization on blood vessels based on EAE or treatment status, no comparisons were made between groups or treatment timelines.

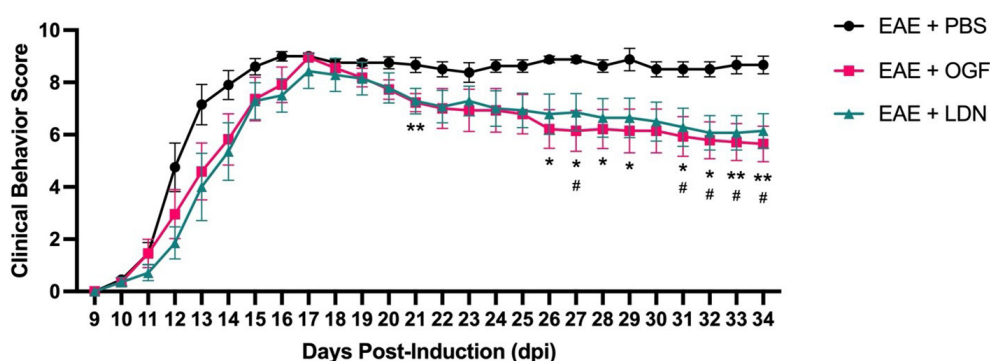


Figure 1. The effect of therapeutic OGF or LDN treatment on behavior in Ch-EAE mice. Mice were treated with 10 mg/kg OGF (n=11 until 21 dpi, n=7 22-34 dpi), 0.1 mg/kg LDN (n=7), or PBS (n=10 until 21 dpi, n=4 22-34 dpi), beginning on the second consecutive day mice showed behavioral signs of EAE (11-12 dpi). Mean clinical behavior scores for OGF or LDN treated mice were significantly lower than PBS treated mice by the end of the experiment. Data were analyzed using a repeated measures mixed-effects analysis of variance with Dunnett's multiple comparisons correction. EAE + OGF group significantly different from EAE + PBS group at $p<0.05$ (*), $p<0.01$ (**). EAE + LDN group significantly different from EAE + PBS group at $p<0.05$ (#).

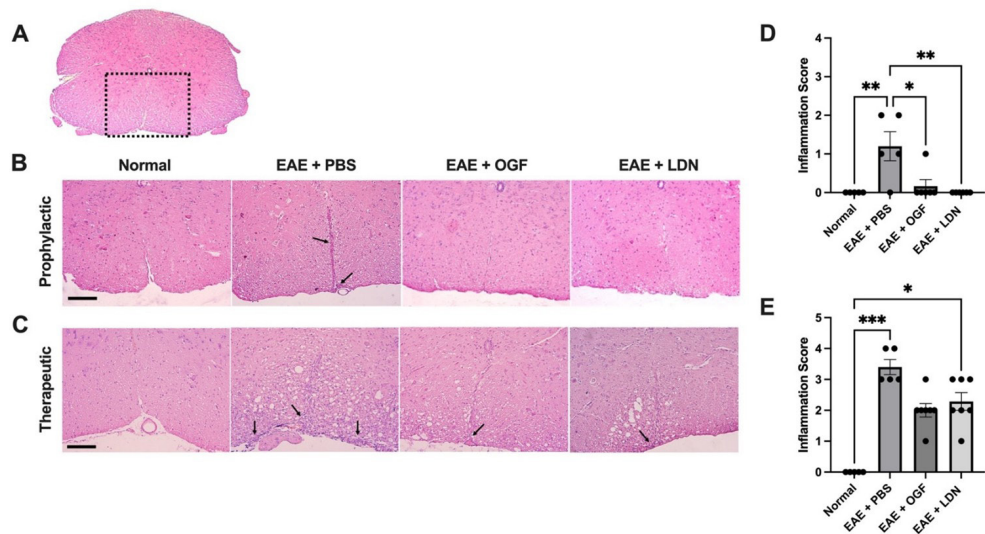


Figure 2. Spinal cord inflammation. **(A)** Hematoxylin & eosin (H&E) stained lumbar spinal cord with selected area shown in C and D (black box). Representative images of H&E-stained spinal cords of normal (non-EAE), EAE + PBS, EAE + OGF, and EAE + LDN mice treated prophylactically for 10 days **(B)** or therapeutically for 3 weeks **(C)**. Arrows indicate sites of immune cell infiltration. Scale bar: 50 μ m. **(D)** Spinal cord inflammation scores comparisons (mean $\pm$ SEM) for prophylactically treated mice and **(E)** therapeutically treated mice. Data were analyzed using Kruskal-Wallis tests with Dunn's multiple comparisons correction. $P<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). $N=5-7$ per group.

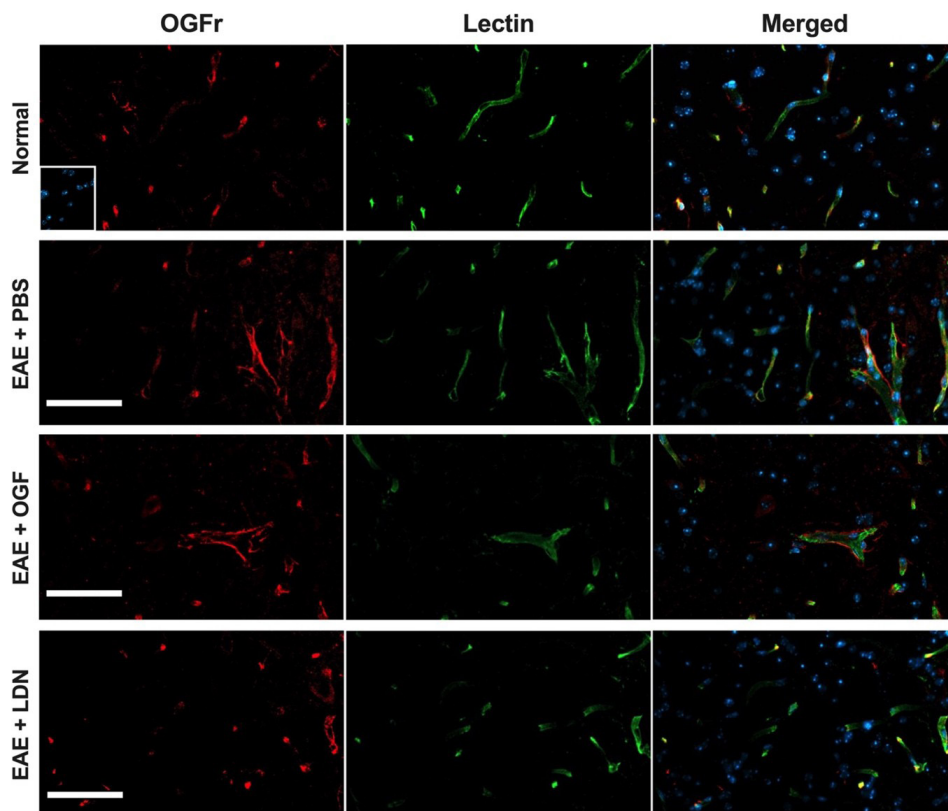


Figure 3. Colocalization of OGFr and endothelial cells. Representative images from OGFr (left) and tomato lectin (middle) immunostained lumbar spinal cords (taken from region outlined in Figure 2A) from normal (non-EAE), EAE + PBS, EAE + OGF, and EAE + LDN prophylactically treated mice. Right column: merged OGFr and lectin with DAPI nuclear counterstain (cyan color). Inset: secondary antibodies only. Scale bar: 50 μ m.

Therapeutic and prophylactic treatment with OGF or LDN limit angiogenesis

To investigate whether OGF or LDN treatment affected angiogenesis, lumbar spinal cords of mice from the prophylactic and therapeutic treatment timelines were stained with tomato lectin and blood vessel density was measured in the grey matter (GM) and white matter (WM) (**Figures 4A-4B**; representative images from region outlined in **Figure 2A**). Analysis of spinal cords from the prophylactic timeline showed no difference between groups for GM lectin % area, but WM lectin % area was significantly greater in EAE + PBS

mice compared to normal, EAE + OGF, and EAE + LDN mice (all $p < 0.0001$) (**Figure 4C**). Prophylactically treated EAE + OGF and EAE + LDN mice had similar WM lectin % area ($p = 0.99$). Analysis of spinal cords from the therapeutic timeline also showed no difference in GM lectin % area (**Figure 4D**). All therapeutically treated EAE groups had significantly greater lectin % area compared to non-EAE controls at 34 dpi (all $p < 0.0001$); however, OGF and LDN treated mice had reduced blood vessel density compared to PBS treated EAE mice (EAE + OGF: $p = 0.05$; EAE + LDN: $p < 0.05$). Therapeutically treated EAE + OGF and EAE + LDN mice had similar WM lectin % area ($p = 1$).

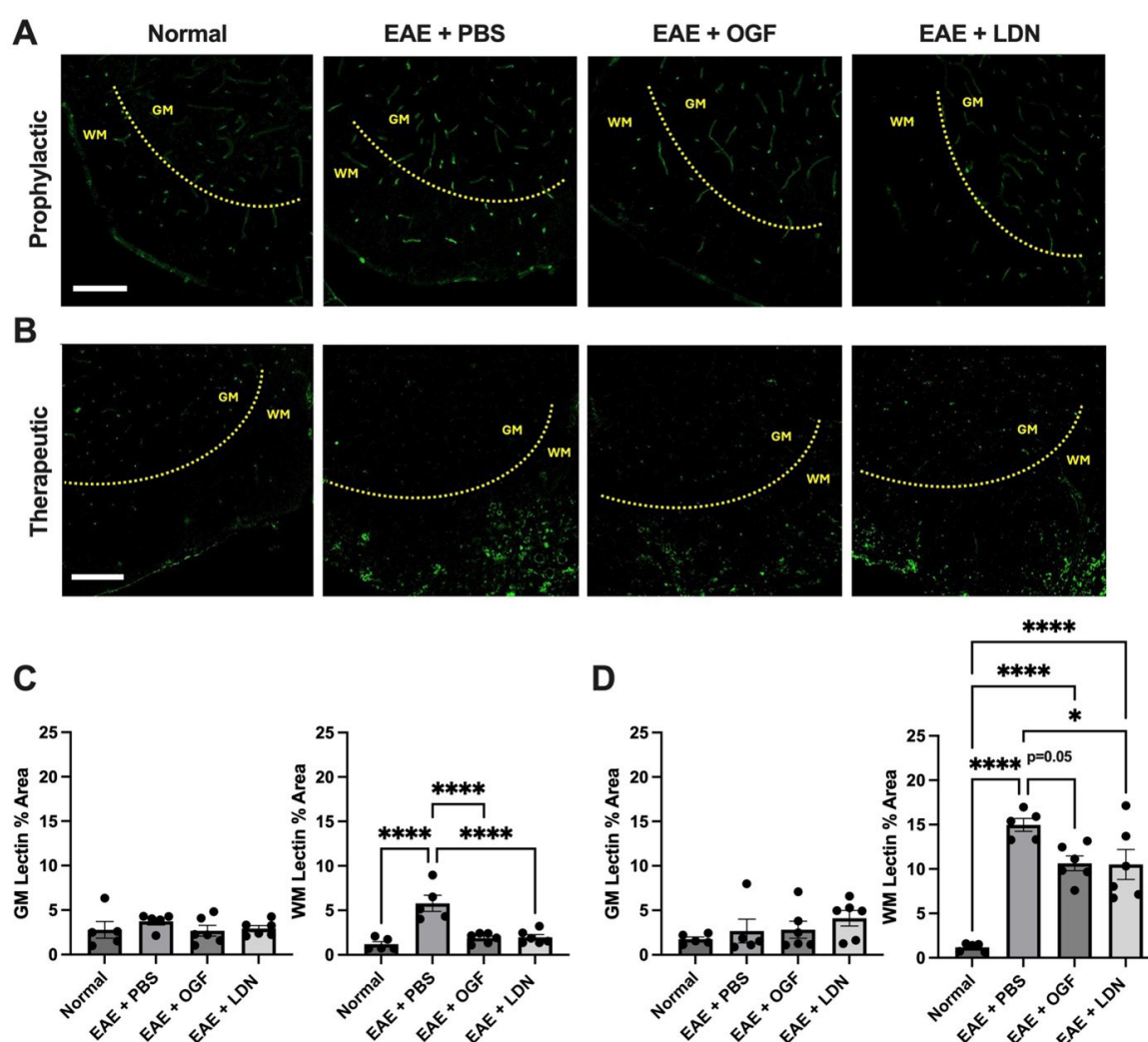


Figure 4. Spinal cord blood vessel density. **(A)** Representative images of lectin-immunostained lumbar spinal cords of normal (non-EAE), EAE + PBS, EAE + OGF, and EAE + LDN mice treated prophylactically or **(B)** therapeutically (taken from region outlined in Figure 2A). Yellow dashed lines approximate the border between grey matter (GM) and white matter (WM). Scale bar: 100 μ m. **(C)** Blood vessel density comparisons in the GM and WM of prophylactically treated or **(D)** therapeutically treated mice. Percent area of lectin staining in two-100 μ m regions of interest was averaged for each mouse, and groups were compared for significant differences using one-way ANOVAs with Tukey's multiple comparisons correction. $P < 0.05$ (*), $p < 0.0001$ (****). $N = 5-6$ per group.

Discussion

OGF or LDN administration ameliorated EAE clinical behavior after approximately 2 weeks of treatment and persisted to the end of the experiment after 3 weeks of treatment (**Figure 1**). All EAE mice experienced the classic “peak disease” rise in clinical scores at ~16-17 dpi, after which OGF and LDN treated mice began to show behavioral improvement and PBS treated mice had a subtle remission followed by partial relapse and stabilization, as is characterized in Ch-EAE [25]. The most valuable metric of therapeutic treatment in Ch-EAE is the final clinical score, which was significantly lower for OGF and LDN treated mice compared to PBS treated mice. One limitation of this study is that, since mice in the prophylactic cohort were euthanized prior to disease onset (10 dpi), clinical scores were not observable in this cohort. However, since 29 of 30 immunized mice (97%) in the therapeutic cohorts developed EAE, we are confident that mice in the prophylactic cohort would have developed a similar induction rate. Additionally, due to the early termination of one therapeutic cohort, there were fewer animals to compare after 21 dpi. This likely reduced statistical power for between-groups comparisons of clinical behavior during 22-34 dpi; however, this makes it even more encouraging that there were significant differences in OGF and LDN treated mice compared to EAE + PBS mice during this period.

We observed significantly greater spinal cord inflammation in prophylactic EAE + PBS mice compared to non-EAE controls, and inflammation was ameliorated in OGF and LDN treated mice at 10 dpi (**Figure 2D**). EAE + PBS mice also had significantly greater inflammation than controls at 34 dpi (**Figure 2E**). Therapeutically treated EAE +OGF and EAE + LDN mice had greater inflammation scores than controls and had an insignificant reduction in inflammation compared to EAE + PBS mice. Taken together, these data suggest that 1) prophylactic OGF or LDN limits CNS infiltration by immune cells prior to symptom onset and 2) therapeutic treatment beginning after symptoms appear is not sufficient to reverse prior immune cell infiltration but may limit ongoing infiltration occurring after onset. Another study limitation is that hematoxylin-labeled cells could include CNS-native pro-inflammatory macrophages and microglia or remyelinating glial cells. While immunolabeling is necessary to determine the specific identities of hematoxylin-labeled cells, this method is commonly used to assess overall infiltration of numerous cell lineages (T- and B-cells) [23,26]. Previous reports also suggest that the OGF-OGFr axis reduces microglial and peripheral lymphocyte activation, providing another potential explanation for the present study's findings [22,27]. To further elucidate the potential targets of the OGF-OGFr axis in EAE, we investigated spinal cord vasculature.

OGFr was present on tomato lectin-labeled endothelial cells that comprise spinal cord blood vessels (**Figure 3**). OGFr is ubiquitously expressed in tissues from all three germ layers in the human, mouse, and rat and has previously been colocalized to T-cells and astrocytes *in vitro* [28,29]. Further evidence is

needed to determine if the OGF-OGFr axis acts directly on endothelial cells to limit proliferation, but the discovery of the receptor localized to endothelial cells supports the possibility of this mechanism. Another possibility is that the OGF-OGFr axis inhibits the proliferation of VEGF-secreting inflammatory cells (e.g., T- and B-cells, activated astrocytes), which would indirectly reduce angiogenesis. Future studies examining the production of VEGF by these cell types in response to OGF or LDN treatment would help deduce the site(s) of action for the OGF-OGFr axis in EAE.

This study presents the first evidence that OGF-OGFr modulation limits angiogenesis in EAE mice. Prophylactic treatment with OGF or LDN significantly reduced white matter (WM) blood vessel density immediately prior to disease onset in EAE mice (**Figure 4C**). All EAE mice had significantly increased WM blood vessel density at 34 dpi compared to controls; however, OGF and LDN treated mice had reduced WM blood vessel density compared to PBS treated mice (**Figure 4D**). In both treatment paradigms, grey matter (GM) blood vessel density was not different between groups. These results corroborate and extend reports of angiogenesis occurring in the WM, but not GM, of EAE mouse spinal cords at 4, 7, and 14 dpi [8]. While GM pathology has been observed in MS and EAE, it is likely that WM inflammation and demyelination predominates in EAE [30,31].

Although the result of OGF-OGFr association is well understood, less is known about how this mechanism fits into complex pathophysiological processes like those attributed to multiple sclerosis. OGF production is paracrine and autocrine, suggesting that cells may be able to self-regulate or regulate the proliferation of nearby cells and tissues [17]. OGF enters the cell *via* clathrin-mediated endocytosis and binds to OGFr located on the nuclear membrane [32]. The OGF-OGFr complex is shuttled to the nucleus by the interaction of OGFr's three nuclear localization signals (NLS) with karyopherin β and Ran [33]. In the nucleus, OGF upregulates p16 and p21 cyclin-dependent kinases, halting the cell cycle at the G₀/G₁ phase and inhibiting cellular replication [33]. The OGF-OGFr axis does not induce apoptosis and is therefore reversible and not cytotoxic. Serum OGF levels are reduced in persons with MS and EAE mice compared to controls, suggesting OGF-OGFr axis dysregulation in these disease states [34,35]. Furthermore, serum OGF has an inverse relationship with serum VEGF [36]. It is possible that OGF-OGFr dysregulation is a factor promoting the imbalanced pro-angiogenic activity in MS and EAE.

Angiogenesis has been implicated in numerous cancers and neurodegenerative diseases like Parkinson's and Alzheimer's, in addition to MS. OGF and LDN treatment reduce tumor burden in some mouse cancer models, and LDN has been shown to improve symptoms of cancer and fibromyalgia (an autoimmune disease) in human patients [37-39]. These previous findings suggest that the OGF-OGFr axis may play a role in the pathophysiology of other neurodegenerative and immune-related diseases, broadening the implications of the present study.

Conclusion

In this study, we found OGFr localized to endothelial cells in the EAE mouse spinal cord. Prophylactic treatment with OGF or LDN limits angiogenesis and immune cell infiltration prior to disease onset, while therapeutic treatment with OGF or LDN ameliorates disease behavior and reduces angiogenesis after 3 weeks of treatment. Therefore, the OGF-OGFr axis may exert its protective effects by limiting angiogenesis in EAE. Future research is needed to determine the specific sites of action for this anti-angiogenic activity.

Conflicts of Interest

The authors report no conflicts of interest.

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