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Original Research

Enkephalin Treatment Reduces Elevated Brain Glia in Mice With Experimental Autoimmune Encephalomyelitis

Rashmi Kumari[1], Ian S. Zagon[1], Patricia J. McLaughlin[1,*]

[1]Department of Neuroscience and Experimental Therapeutics, Penn State University College of Medicine, Hershey, PA 17033, USA
*Correspondence: pxm9@psu.edu (Patricia J. McLaughlin)
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Abstract

Background: Multiple sclerosis (MS) is a chronic, autoimmune disorder that culminates in severe motor and cognitive deficits. The progression of the disease is associated with inflammation and demyelination of the brain and spinal cord. Activation and proliferation of glia, including astrocytes and oligodendrocytes, in the brain are hallmarks. Persons with MS (PwMS) have significantly low levels of the neuropeptide [Met^5]-enkephalin (Met^5-Enk), also termed Opioid Growth Factor (OGF) that is an inhibitory growth factor and modulates cellular homeostasis. Treatment with low doses of naltrexone, an opioid antagonist that blocks the receptor OGFr, is reported to decrease inflammation, neutralize pro-inflammatory cytokine secretion, and stabilize disease progression. Intermittent blockade of OGFr with low doses of naltrexone stimulates serum enkephalin levels. In this investigation, brains of mice with experimental autoimmune encephalomyelitis (EAE) were treated with low dose naltrexone (LDN) or OGF/Met-Enk and examined for reactive astrocytes and oligodendrocytes. **Methods:** Female C57BL/6J mice were inoculated with myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) to induce EAE. At the initial appearance of clinical behavioral symptoms, mice were randomized to receive 10 mg/kg [Met^5]-enkephalin (OGF/Met-Enk), 0.1 mg/kg naltrexone (LDN), or 0.1 mL phosphate-buffered saline (PBS) daily. At 35 days post EAE induction, mice were euthanized, and the brains were removed and prepared for immunohistochemistry. The corpus callosum, subventricular zone, and dentate gyrus were stained with glial fibrillary acidic protein (GFAP) and adenomatous polyposis coli protein (APC) antibodies to detect astrocytes and oligodendrocytes, respectively. **Results:** The number of reactive astroglia in EAE brains was elevated relative to the number in normal mouse brains. Daily treatment with OGF/Met-Enk reduced GFAP-positive cells in all 3 brain regions, and LDN reduced astrocyte number in the corpus callosum and subventricular zone. Astrocytes in treated EAE mice had process lengths that were comparable with those in normal mice, suggesting less reactivity. The numbers of oligodendrocytes in the corpus callosum of EAE+LDN mice were significantly reduced relative to EAE+PBS tissues. **Conclusions:** These results demonstrate quantitative changes in reactive glia in EAE mouse brains following treatment with OGF/Met-Enk and LDN and corroborate other studies showing that OGF/Met-Enk and LDN treatment increase plasma enkephalins, reduce inflammation and decrease pro-inflammatory cytokines.

Keywords: astrocytes; oligodendrocytes; subventricular zone; dentate gyrus; corpus callosum; [Met^5]-enkephalin

1. Introduction

Multiple sclerosis (MS) is a chronic and debilitating autoimmune disease of the central nervous system (CNS) that affects approximately 1 million people in the United States and nearly 3 million individuals worldwide [1]. MS occurs in chronic, progressive, and relapse-remitting forms, and can be characterized by astrocyte reactivity leading to inflammation and recruitment of activated T cells to the CNS that in turn promote demyelination and neurodegeneration [2,3,4,5]. Current U.S. Food and Drug Administration (FDA)-approved therapies target one or more symptoms, have severe side-effects, are expensive, and have low compliance rates [1]. Furthermore, many disease-modifying therapies do not alter the underlying pathophysiology of persons with MS (PwMS) [6].

Studies have reported that individuals with chronic diseases have altered levels of Opioid Growth Factor (OGF), chemically termed [Met^5]-enkephalin (Met-Enk) [7,8,9,10]. In comparison to baseline levels in normal patients that range from less than one pmol/mL [9] to approximately 12–20 pg/mL [10], PwMS have reduced levels [8]. In a study of 55 PwMS, behavioral deficits as monitored by slow walking times were correlated with fatigue, anxiety, depression, and pain; a subset of these patients had depressed or undetectable levels of serum enkephalin in enzyme-linked immunosorbent assay (ELISA) studies [11].

Our laboratory has established mouse models of MS, experimental autoimmune encephalomyelitis (EAE), for chronic, progressive and relapsing-remitting forms [12,13,14]. Although animal models do not recapitulate the etiology of MS, the pro-inflammatory diseases are similar, as the levels of interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) are upregulated in EAE and MS, and are characterized by CNS demyelination and neurodegeneration [13,14,15,16].

In vitro data using purified astrocyte cultures revealed that OGF/Met-Enk inhibited growth in a dose-dependent (10^{-4} M–10^{-8} M) and receptor-mediated manner; the num-

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ber of astrocytes was reduced up to 40% within 72 hr of treatment with 10^{-6} M OGF relative to sterile water treated control cultures suggesting that astrocytic proliferation is very responsive to the inhibitory action of OGF/Met-Enk [5]. Molecular-based investigations of T-cells grown in culture revealed that OGF/Met-Enk and OGFr were required for this inhibition, as knockdown of OGFr eliminated the inhibitory activity of OGF/Met-Enk, whereas siRNA knockdown of classical opioid receptors (mu, delta, kappa) had no effect on cell replication [17].

Systemic treatment to elevate OGF/Met-Enk in the relapse-remitting model of EAE revealed that sustained exogenous treatment resulted in substantial beneficial effects. Cohorts of mice with relapsing-remitting EAE, established by injections of proteolipid protein, had more relapses and elevated levels of Iba-1 expression and CD3+ reactive cells that stain microglia/macrophages and T and natural killer (NK) cells, respectively. OGF/Met-Enk treatment reduced reactive cells in the spinal cord and inhibited proliferation of glia and T lymphocytes. Dual-labeled Ki67 and glial fibrillary acidic protein (GFAP) cells were significantly decreased relative to untreated relapsing-remitting (RR)-EAE mice [12]. Direct injections of OGF/Met-Enk prevented the expression of disease in 37% of mice, reduced the severity and disease indices of other EAE mice, increased remission 6-fold in OGF/Met-Enk-treated mice, and reduced astrocyte activation [12].

Previous studies of mice with confirmed chronic progressive EAE and treated with OGF/Met-Enk (10.0 mg/kg [Met^5]-enkephalin) had reduced clinical behavioral scores and less demyelination [13,18]. Specific cell counts in the lumbar spinal cord revealed elevated numbers of astrocytes and microglia/macrophages in mice with disease receiving saline injections (vehicle) relative to normal mice, whereas mice with established disease but treated with OGF/Met-Enk had significant reductions in microglia within 5 days of treatment and astrocyte number was decreased 5-fold within 10 days in mice treated with OGF/Met-Enk [13].

Investigations in humans have reported that two opioid peptide proteases, cluster of differentiation (CD)10 and CD13, that break down OGF/Met-Enk are increased in the neutrophil cell population in patients with active MS, but are decreased in patients undergoing remission [19]. Studies have shown an increase in the enzymes CD10 (neutral endopeptidase-NEP; EC 3.4.24.11) and CD13 (aminopeptidase N; AP-N, EC 3.4.11.2) in MS patients during the course of relapses, but low expression of these peptidases during remission. Because these ectoenzymes are enkephalin-degrading, it can be postulated that enkephalins, which inhibit cell proliferation, would be decreased, thus resulting in uncontrolled T cell proliferation and further exacerbation of MS and neurodegeneration. Together, data on decreased enkephalins and increased enkephalinases in MS patients establish an important link between opioid peptides and MS.

To continue examination of the neuropathology in EAE, mice with chronic EAE and treated with either OGF/Met-Enk or low dose naltrexone (LDN) were examined for amelioration of astrocyte and oligodendrocyte number in 3 regions of the brain. Immunohistochemical studies with antibodies to GFAP in the dentate gyrus, subventricular zone, and corpus callosum, and antibodies to adenomatous polyposis coli protein (APC) for oligodendrocytes of the corpus callosum. These brain regions were selected based on their abundance of glial cells, interneuronal connections, and the frequency of astrogliosis in neurodegenerative diseases. The dentate gyrus aids in processes of learning and memory, the subventricular zone has neural progenitor and stem cells that propagate new neurons and glia for damaged regions of the brain, and the corpus callosum contains numerous axons involved with integration of information and cognitive processing.

2. Materials and Methods

2.1 Animal

Nine 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 ± 0.5 °C with a relative humidity of 50 ± 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. Completed ARRIVE 2.0 guidelines are included in the **Supplementary Material**.

2.2 Induction and Confirmation of EAE

As previously described [17], EAE was induced by subcutaneous injections (0.1 mL) of emulsified myelin oligodendrocyte glycoprotein ($MOG_{35–55}$ (400 µg)) dissolved in complete Freund's adjuvant (#EK-2110, Hooke Laboratories, Lawrence, MA, USA). Two intraperitoneal injections (0.1 mL) of 110 ng pertussis toxin (PTX) (Cat. # I-181; List Biologicals, Campbell, CA, USA) in sterile phosphate-buffered saline (PBS) were injected on days 0 and 1. $MOG_{35–55}$ was synthesized at Penn State College of Medicine Core Facility and purified and validated on site. Mice were lightly anesthetized with 3% isoflurane (Cat. # 50989-150-15, Vedco, Inc., St. Joseph, MO, USA) for $MOG_{35–55}$ injections. Normal mice received equal volumes of sterile PBS and served as controls.

Clinical behavior scores were used to confirm the presence of EAE [13,18]. Beginning in the second week following induction, mice were observed daily for clinical signs of EAE such as dragging tail, wobbly gait, or limb paralysis.

2.3 Drug Treatment

Beginning on day 11 post $MOG_{35–55}$ inoculation, mice received daily intraperitoneal injections of 0.1 mg/kg naltrexone (EAE+LDN), 10 mg/kg enkephalin

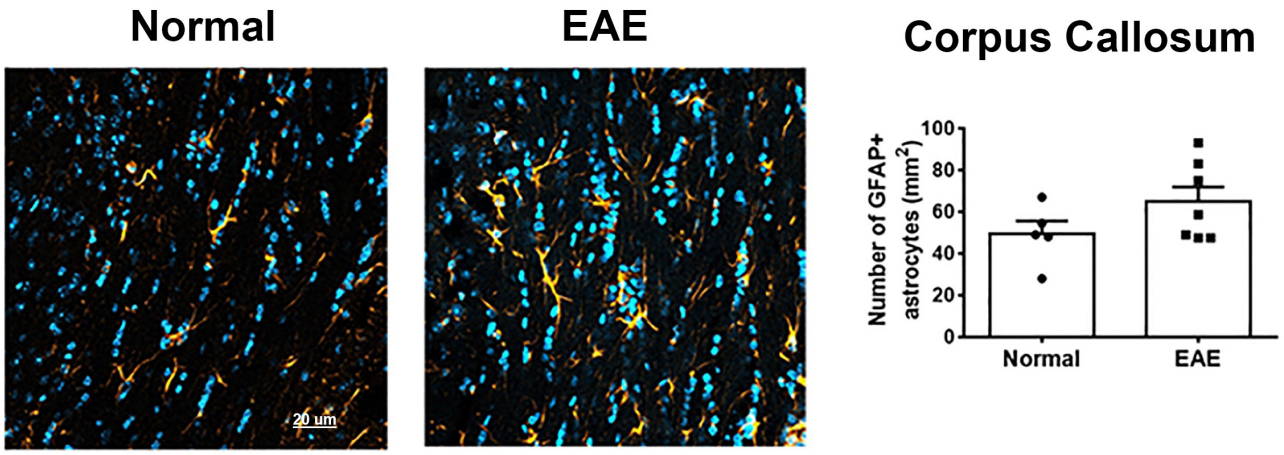


Fig. 1. Brain sections of corpus callosum from normal female C57BL/6J mice and those induced with myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) to develop experimental autoimmune encephalomyelitis (EAE), and stained with glial fibrillary acidic protein (GFAP) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Histograms represent means ± standard error of the mean (SEM) for each section counted in 3 or 4 mice for all figures. 20× magnification. Scale bar = 20 µm.

(EAE+OGF/Met-Enk) or 0.1 mL PBS (EAE+PBS). Normal mice received daily injections of PBS. Treatment continued until 35 days post $MOG_{35–55}$ inoculation at which time mice were deeply anesthetized with 3% isoflurane and euthanized using the Euthanex carbon dioxide chamber (Perotech) with a displacement rate of 30-70% per minute, followed by intracardial perfusion with PBS and then with 4% paraformaldehyde (PFA). Euthanasia is confirmed by toe pinch (no reflex), visualization of the eye (turned white), and final removal of the brain. Brains were removed and either post-fixed in 4% PFA overnight and embedded in paraffin or frozen in isopentane chilled on dry ice and prepared for cryosectioning.

2.4 Tissue Preparation, Antibody Staining, and Immunofluorescence

Frozen brain sections (16 µm) were collected at the level of the striatum and hippocampus and stained with antibodies to GFAP to assess the reactive astrocytes. Tissues were post fixed with 4% paraformaldehyde for 10 min, blocked with 10% normal goat serum/0.3% Triton/1× PBS for 1 h, and incubated with mouse monoclonal GFAP antibody (Cat. # MAB 360; dilution, 1:500; Millipore Sigma, Burlington, MA, USA) at 4 °C overnight. The next day, sections were incubated with goat anti-mouse antibody conjugated to Alexa Fluor™-546 (orange red, dilution, 1:500) for 1 h at room temperature. For double staining of Ki67 and GFAP, fresh frozen brain sections were blocked with 10% NGS /0.3% Triton/1× PBS/1% BSA/0.3M glycine for 1 h before incubation with rabbit polyclonal Ki67 antibody (Cat. # PA5-16446, Invitrogen, dilution 1:100, Carlsbad, CA, USA) and GFAP. Tissues were incubated overnight at 4 °C, washed, and incubated with secondary antibodies Alexa Fluor™ 488 and Alexa Fluor™ 568 (dilution 1:500) for 1 h at room temperature.

For mature oligodendrocyte immunostaining, frozen sections were thawed, washed twice with 1× PBS for 5 minutes each, post-fixed with 4% paraformaldehyde for 10 minutes, and washed in 1× PBS twice for 5 minutes. Antigen retrieval was performed by boiling tissue for 30 min in 10 mM sodium citrate at pH 6.0, and permeabilized for 30 min with 0.5% Triton in 1× PBS. Sections were blocked as stated above and incubated with mouse monoclonal anti-APC antibody (CC1) (Cat. # ab16794; dilution, 1:100; Abcam, Cambridge, UK) overnight at 4 °C. The next day, sections were washed with 0.3% Triton/1× PBS 3 times for 15 min with gentle agitation before addition of Alexa Fluor™-488 (dilution, 1:500) was applied. Following addition of secondary antibodies, tissues were washed and then incubated with 4′,6-diamidino-2-phenylindole (DAPI, dilution, 1:5000) for 10 min, and washed and mounted with Aqua Poly/Mount (Cat. #18606, Polysciences, Warrington, PA, USA). Slides were stored at 4 °C prior to imaging using a Zeiss LSM 900 confocal microscope (Zeiss, Oberkochen, Germany). Negative controls were tissues without primary antibodies.

2.5 Confocal Microscopy and Quantitation of Glial Cells

The number of reactive astrocytes was quantified in the corpus callosum, subventricular zone, and dentate gyrus region and oligodendrocytes in the corpus callosum were counted in 2 grids/section and 3 sections/brain, from 3–4 mice in each group. The images were captured at similar settings (512 × 512 mm^2, 20× magnification, 0.2% laser power) in all the groups. The number of GFAP-positive reactive astrocytes and oligodendrocytes was counted using the NIH ImageJ software 2.0 (National Institutes of Health, Bethesda, MD, USA) threshold method as previously described [20]. In addition, process lengths for approximately 15 astrocytes in each group in the dentate gyrus were mea-

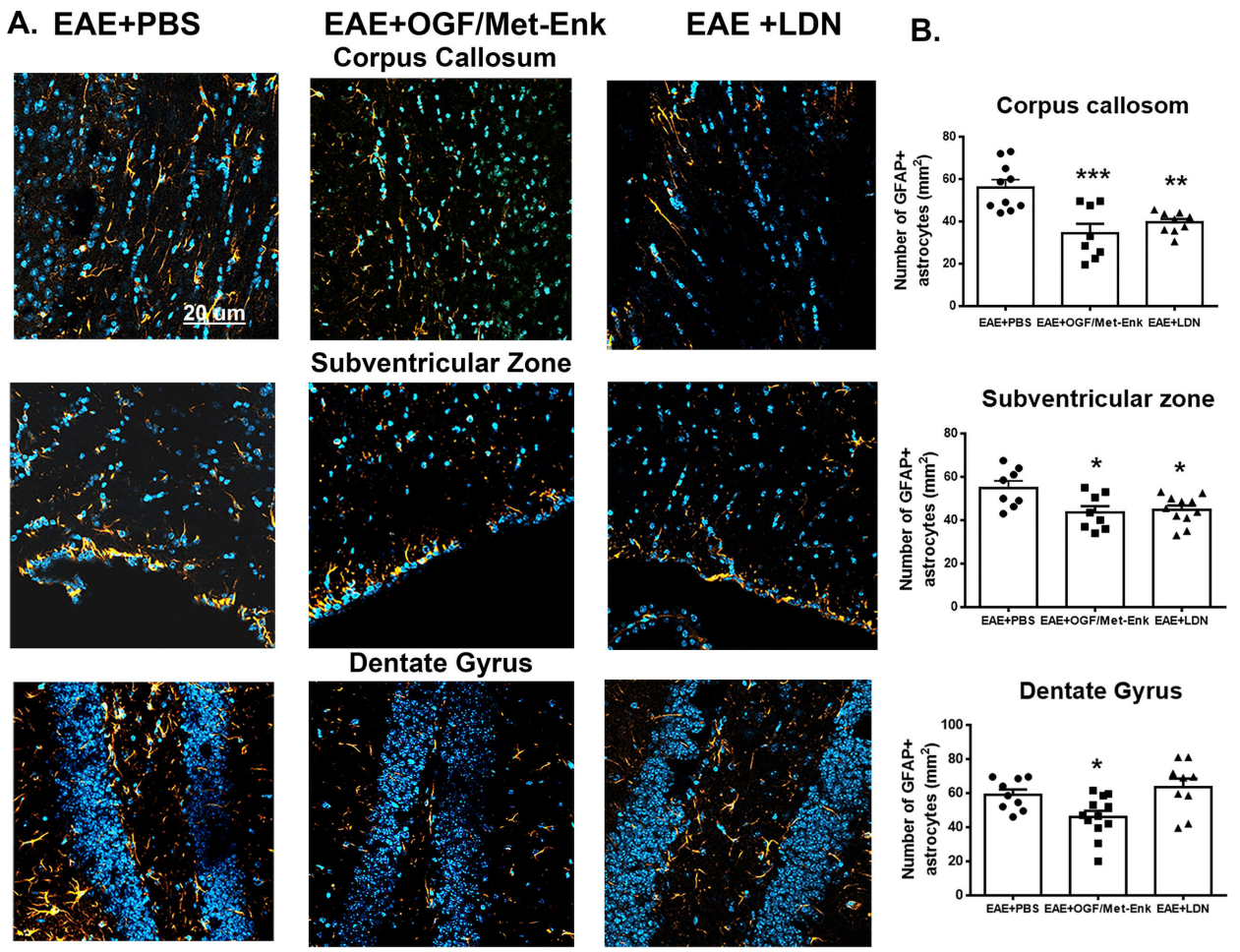


Fig. 2. [Met^5]-enkephalin and LDN treatment reduces the number of reactive astrocytes in different brain region of EAE mice. (A) Tissue sections of corpus callosum, subventricular zone, and dentate gyrus from mice with EAE and treated with phosphate-buffered saline (EAE+PBS), 10 mg/kg [Met^5]-enkephalin (EAE+OGF), and 0.1 mg/kg naltrexone (EAE+LDN) and stained with antibodies to GFAP and counterstained with DAPI. Scale bar = 20 µm. (B) The numbers of GFAP-positive cells were counted in both hemispheres of brains from 3 sections per mouse and 3–4 mice per treatment group. Points on the histograms represent biological replicates, that is, counts from each hemisphere for 3-4 mice per group. Cell counts were converted to mm^2. Histograms represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus EAE+PBS group. PBS, phosphate-buffered saline; OGF, Opioid Growth Factor; LDN, low dose naltrexone.

sured using ImageJ software. For double-labeled immunostaining, color deconvolution was applied to separate the image into three colors, and the threshold of the image was then adjusted and converted into a binary image to analyze the number of particles based on the size and color. The particle size, image size, and DPI remained unchanged for all images to exclude any background particles.

2.6 Statistical Analyses

Cell count data and process lengths are presented as means ± standard error of the mean (SEM). Numbers of cells were counted in both hemispheres of brains from 3 sections per mouse, averaged as technical replicates within a single animal; 3–4 mice per treatment group were averaged. The statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, LLC, San Diego, CA, USA) and data were analyzed using one-way analysis of variance, followed by Tukey's multiple comparisons to compare the means amongst the groups. The significance was set at $p < 0.05$.

3. Results

3.1 Astrogliosis in the Brains of Mice With EAE

Mice inoculated with $MOG_{35–55}$ demonstrated clinical signs of motor deficits by day 11 [18]. Astrogliosis was noted in all areas of the EAE brain. Representative images of the corpus callosum from normal and EAE mice are presented in Fig. 1 and show increased levels of reactive astrocytes in tissue from EAE mice.

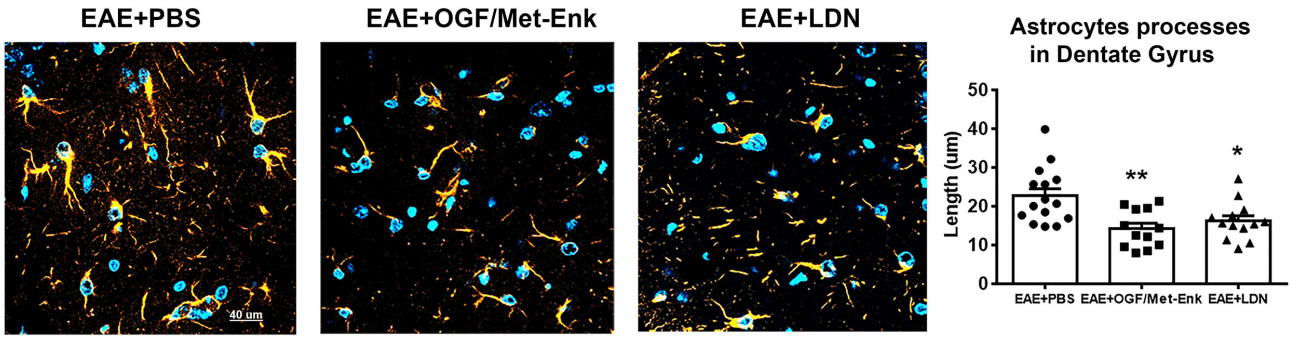


Fig. 3. Morphological images of reactive astrocytes from the dentate gyrus region of brains from cohorts of mice receiving PBS (EAE+PBS), 10 mg/kg [Met^5]-enkephalin (EAE+OGF), and 0.1 mg/kg naltrexone (EAE+LDN). Length of reactive astrocyte processes were measured by ImageJ software and presented as means ± SEM for approximately 15 astrocytes in the dentate gyrus of each cohort. Significantly different from EAE+PBS at *$p < 0.05$ or **$p < 0.01$. 40× magnification. Scale bar = 40 µm.

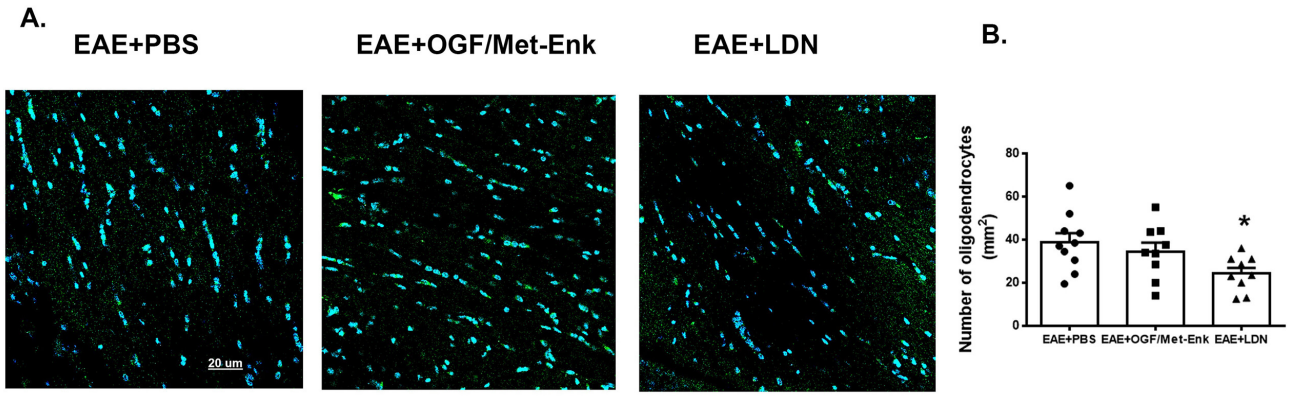


Fig. 4. Mature oligodendrocytes decreased in corpus callosum in EAE mice post LDN treatment. (A) Corpus callosum sections from mice with EAE and treated with PBS (EAE+PBS), 10 mg/kg [Met^5]-enkephalin (EAE+OGF), and 0.1 mg/kg naltrexone (LDN) and stained with antibodies to APC and counterstained with DAPI to reveal oligodendrocytes. Scale bar = 20 µm. (B) The numbers of APC-positive oligodendrocytes were counted in both hemispheres of the corpus callosum, 3 sections/mouse, 3–4 mice per treatment group. Data within the grid were converted to mm^2. Histograms represent means ± SEM. *$p < 0.05$ versus EAE+PBS group. APC, adenomatous polyposis coli protein.

3.2 Enkephalin Decreases the Number of GFAP-Positive Astrocytes in the Corpus Callosum, Subventricular Zone, and Dentate Gyrus in EAE Mice Receiving OGF/Met-Enk or LDN

Images of reactive astrocytes in the corpus callosum, subventricular zone and dentate gyrus from mice in the EAE+PBS, EAE+OGF/Met-Enk, and EAE+LDN cohorts are presented in Fig. 2a. Cell counts of reactive astroglia in these regions of the brain revealed that systemic OGF/Met-Enk decreased astrocytes in all 3 regions, and LDN significantly decreased astrocytes in the subventricular zone and corpus callosum (Fig. 2b). Within the grid area, approximately 56 reactive astrocytes were counted in the corpus callosum of EAE+PBS mice in comparison to less than 40 reactive astrocytes in the EAE+OGF/Met-Enk and EAE+LDN treated mice. Within the subventricular zone, there were approximately 55 GFAP+ stained cells in comparison to a 21% decrease ($p < 0.05$) in reactive astrocytes. Within the dentate gyrus, there were approximately 60 reactive astrocytes per grid, and a 23% decrease ($p < 0.05$) in numbers only following OGF treatment.

A magnified image of reactive astrocytes from the dentate gyrus is presented in Fig. 3. Images of the morphology of reactive astrocytes in the EAE brain have extended processes suggesting a pathological state in comparison to cells in the EAE+OGF/Met-Enk treated brain tissue. Changes in the morphology of astrocytes have been well documented as an indication of their roles in neuroprotection and neurodegeneration [21,22].

3.3 Enkephalin Decreases the Number of APC-Positive Oligodendrocytes in the Corpus Callosum of EAE Mice Treated With LDN

Mature oligodendrocytes, stained with CC1, were counted only in the corpus callosum, where they were most prominent in their role in myelination (Fig. 4a). Cell counts

revealed that the number of mature oligodendrocytes/mm^2 was reduced 25% ($p < 0.05$) in mice receiving LDN treatment (Fig. 4b).

4. Discussion

Several million PwMS are living with the disorder and many may have been diagnosed as early as age 20 [1]. MS has a multifactorial etiology. Symptoms and signs involve the immune system and astrogliosis [3,4]. Neural-related disorders that invoke brain damage such as MS result from reactive astrogliosis and the resultant cytokine production [22,23,24,25,26]. Reactive astrocytes can lead to inflammation and loss of axons and neurons which contributes to demyelination [22,23,24,25]. Further damage to the blood brain barrier occurs with changes in astrocyte morphology particularly in regard to neuromyelitis optica [26]. Astrocyte morphology remains a strong clinical sign during early and advanced stages of MS. Disease-modifying treatments are only partially successful in targeting astrocyte proliferation [24], as the cause for the proliferation is not reduced by the therapy. Other studies imply that oxidative stress alters the number and function of neurons and glia in MS, leading to impaired mitochondria and release of free radical species [25,27]. This line of thought toward the mechanism of MS may provide new areas of research.

The current study examined levels of reactive glia in specific areas of the brain that are prone to expressing astrogliosis [22,26]. The subventricular zone and dentate gyrus were very responsive to treatment that depressed astrocyte number. The corpus callosum contains millions of axons that bridge the right and left hemispheres and through communication between both sides of the brain coordinate motor, sensory, and cognitive functions. Because mature oligodendrocytes support this axonal myelination the area was chosen for analysis. In mice with EAE, the number of oligodendrocytes was increased, and decreased following approximately 3 weeks of treatment with LDN. There was no distinction between mature (CC1-positive) and precursor cells. Because reactive glial cells are decreased following LDN treatment, the number of mature (CC1-positive) cells would be proportionately decreased. Future studies may include differentiating between mature and precursor oligodendrocytes.

The dysregulation of the OGF-OGFr regulatory axis in MS provides a new pathological target of study. Intermittent treatments that block the interaction of OGF/Met-Enk with its nuclear-associated receptor OGFr for a short-period of time (<8 hr) are followed by release of elevated levels of the inhibitory growth factor OGF/Met-Enk [28]. This LDN treatment has been reported to have positive effects on the behaviors and well-being of PwMS [11], as well as reduce inflammation in cancer and other autoimmune disorders [29,30].

5. Conclusion

These studies confirm that enkephalins play a role in astrogliosis observed in mouse models of MS. Treatment with LDN and OGF/Met-Enk that elevate serum levels of OGF/Met-Enk decreased the number of reactive astrocytes in three regions of the mouse brain and oligodendrocytes in the corpus callosum. Treatment with LDN that intermittently blocks interactions between OGF/Met-Enk and OGFr for short periods of time and then stimulates a rebound of endogenous OGF/Met-Enk, an inhibitory growth peptide, was very effective at reducing astrogliosis. In concert with previously published data on the efficacy of LDN for treatment of anxiety, depression, and pain in a variety of autoimmune disorders, as well as its profile of tolerability and safety, and low expense, clinical trials are warranted as a disease-modifying therapy that targets underlying pathophysiology of MS.

6. Limitations

A full study on mechanisms underlying the pathophysiology of EAE/MS was limited by the availability of brain tissue in the experimental groups. Nevertheless, this study provides foundational work on reactive astrocytes and glia in several brain regions of EAE mice, and demonstrates that blockade of the dysregulated OGF-OGFr pathway in EAE can begin to restore brain function.

Availability of Data and Materials

Original data may be available from the corresponding author upon reasonable request.

Author Contributions

RK: Data curation, data analyses; writing – reviewing and editing final draft. ISZ: Supervision, funding acquisition, interpretation of data, writing-reviewing and editing final draft. PJM: Conceptualization, supervision, funding acquisition, writing – original draft, writing – reviewing and editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Penn State University College of Medicine IACUC (Institutional Animal Care and Use Committee) protocol 464834. All procedures were performed in accordance with the guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN52956.

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