Abstract

Background: Relapse-remitting multiple sclerosis is a chronic disorder that affects more than 400,000 individuals in the United States, often reducing their quality of life, increasing medical expenses, and limiting mobility. This study examines modulation of the opioid growth factor (OGF)–OGF receptor (OGFr) axis by low dosages of naltrexone (LDN) as a disease modifying therapy using a mouse model of relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE).

Methods: RR-EAE was induced by immunization of SJL/J mice with proteolipid protein 139-151. After two days of clinical disease, mice were injected intraperitoneally with 0.1 mg/kg naltrexone (LDN) or saline for 40 days. Behavior was observed daily, and periodically, mice were euthanized and spinal cords collected for neuropathological evaluation of glia, T lymphocyte infiltration, and demyelination.

Results: LDN treatment significantly reduced behavioral scores across the 40 day observation period. LDN therapy increased the length of remission, as well as the duration of mild disease. A bimodal distribution of behavioral response to LDN was noted that distinguished “responders” from “non-responders”. Pathological analyses of spinal cord tissue from all LDN-treated mice revealed reductions in the number of inflammatory cells (microglia/macrophages), activated astrocytes, and proliferating cells, as well as decreases in areas of demyelination relative to saline-treated mice with RR-EAE.

Conclusions: These data are the first to demonstrate that modulation of the OGF-OGFr axis by LDN in mice with established RR-EAE is effective at reducing clinical behavior and central nervous system neuropathology.

Keywords: Opioid antagonists; Naltrexone; Endogenous enkephalins; Demyelination; Multiple sclerosis

Introduction

More than 2.3 million individuals worldwide have multiple sclerosis [1], and a majority of the individuals experience the relapse-remitting form. All forms of MS are characterized by progressive neurodegeneration in the spinal cord and brain, resulting in a reduction in the quality of life and increased medical expenditures [2]. Approved therapies often target symptoms of the disease rather than disease-based mechanisms, and thus are not completely effective leading to reduced compliance [1-6].

A novel biological pathway has been identified that regulates homeostasis of replicating cells and tissues [7]. The pathway involves the endogenous opioid termed methionine enkephalin (opioid growth factor, OGF) and its nuclear-associated receptor, OGF receptor (OGFr) [7,8]. Modulation of the pathway can be direct by exogenous administration of OGF peptide or endogenously by stimulating production of peptides and receptors following short-term opioid receptor blockade by naltrexone. The use of low dosages of naltrexone (LDN) to invoke a short duration of opioid receptor blockade markedly inhibits cancer cell replication [9]. LDN treatment changes the course of progressive experimental autoimmune encephalomyelitis (EAE) [10,11]. Previous studies have reported that mice immunized with myelin oligodendrocytic glycoprotein 35-55 (MOG_{35-55}) to establish progressive EAE and injected daily with 0.1 mg/kg NTX beginning at the time of disease induction had delayed onset of clinical disease, as well as reduced severity of behavioral deficits; in some cases the course of EAE was reversed within a few days [10-12]. Neuropathology of the lumbar spinal cord revealed significant reductions in the number of activated astrocytes and regions of demyelination [10,11].

Despite more than 85% of patients presenting initially with relapse-remitting forms of MS [1,2], animal models for this form of MS are not widely used. For those models that have been routinely used in basic science research, review of the data reveal that depending on the source of mice, there are problems with penetrance and consistent expression of behavioral characteristics [13]. In general relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) is induced in mice by immunization with proteolipid protein 139-151 (PLP_{139-151}) [14-17]. The animal response is manifested by proliferation and activation of T-lymphocytes, microglia, and astrocytes, resulting in inflammation, demyelination, and axonal damage, that characterizes a well-defined clinical behavior and neuropathology [13,17]. Some investigators have suggested that the RR-EAE mouse model is inconsistent with human scenarios because not all animals express remissions, and many appear to have a more chronic progressive disorder [13]. Our laboratory has established this model of RR-EAE and initiated treatment at the time of induction before behavioral changes were observed [18]. Daily treatment with exogenous OGF of mice immunized with PLP reduced clinical signs of disease within 9 days of treatment. Median cumulative disease scores of OGF-treated RR-EAE mice were decreased 66% from median behavioral scores of saline-treated RR-EAE mice [18]. Importantly, the number and severity of relapses were limited over the course of 55 days. In recent studies utilizing OGF treatment of established disease, OGF was shown to markedly reduce clinical disease in SJL mice that responded to OGF, reduce relapses, and render many mice in total remission [19].

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A few clinical trials have utilized LDN for treatment of patients with progressive or relapse-remitting forms of MS [20,21]. Chart reviews of patients taking LDN therapy, alone or in combination with other immune modulating drugs such as Copaxone® revealed that patients reported no side effects and some resolution to the nausea and fatigue associated with multiple sclerosis [22].

The present study examined the effects of daily LDN therapy on established RR-EAE disease beginning LDN treatment 2 days after initial clinical signs. The animals were observed daily for 40 days to evaluate relapses and remissions. Periodically, lumbar spinal cord tissue was collected examined histologically to assess the expression of microglia, macrophages, T lymphocytes, activated astrocytes and proliferating cells. Changes in pathology were correlated to behavioral differences.

Methods
Animals and induction of RR-EAE
Female SJL/JOrlCRL mice (6-8 weeks of age) were purchased from Charles River Labs (Wilmington, MA) and housed 5 per cage in a separate room from other rodents, with food and water available ad libitum. Soft food and water packets were placed on the floor of the cages for mice unable to obtain food or water from the standard dispensers. All experiments were conducted in accordance with the National Institutes of Health guidelines on animal care, and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

RR-EAE was induced by immunization with proteolipid-protein 139-151 (PLP139-151) [14,15] in 80 SJ/L/J mice. Mice were inoculated subcutaneously on the back with multiple injections (total volume 300 μl) of an emulsion containing 100 μg PLP139-151 (Peptides International, Louisville, KY) and 250 μg Mycobacterium tuberculosis (H37RA, Difco Laboratories, Detroit, MI) added to 0.15 ml incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO) to make complete Freund’s adjuvant (CFA). The final mixture contained equal volumes of the phosphate-buffered saline with PLP139-151 and CFA. Intraperitoneal (i.p.) injections of pertussis toxin (200 ng) in phosphate buffered saline (List Biological Laboratories, Campbell, CA) were given on days 0 and 2. Animals were lightly anesthetized with 3% isoflurane (Vedco, Inc., St. Joseph, MO) for injections of PLP139-151 and M. tuberculosis, but not for daily therapeutic treatments. 10 mice were not immunized and served as normal controls for comparisons of spinal cord pathology.

Drug treatments
Mice were observed daily for behavior and scored based on the criteria described below [18,19]. Drug treatments were initiated when mice displayed characteristic signs of RR-EAE for 2 consecutive days (=established disease). Animals were randomized to receive daily i.p. injections (0.1 ml) of naltrexone (0.1 mg/kg) (Sigma-Aldrich, Indianapolis, IN) (RR-EAE+LDN; n=40) or sterile phosphate-buffered saline (RR-EAE+Saline; n=40). Injections were given between 0900 and 1000 h, and all animals were weighed weekly in order to adjust drug dosages.

Behavioral observations
Beginning on day 8 after immunization, each mouse was observed daily by two individuals (one observer masked to treatment) and behavior scored by placing each animal on a smooth surface and recording tail tonicity, gait, and righting reflex. To determine limb strength, mice were inverted on a wire grid and observed for their ability to maintain grasp. A modified behavioral scale of 0 to 10 was used in order to record incremental changes in behavioral events [18,19,23]. Each mouse received scores for tail tone, gait, righting reflex, and individual limb tonicity, and the sum of scores was recorded daily. Paralysis of each limb was noted when the limb was unable to support body weight; thus, a mouse could have paralysis in one to four limbs.

Onset of the disease was considered the second consecutive day that a mouse had a behavioral score of 0.5 or greater. Disease severity for each treatment group was the number of days that animals had individual behavioral scores greater than or equal to four and a cumulative disease score for each mouse was the summation of behavioral scores over the 40-day treatment period.

Remissions and relapses
Complete remission occurred when the behavioral score returned to 0.5 or less for two consecutive days. "Mild" disease was characterized by behavioral scores less than or equal to 2.0 for two consecutive days. Partial remission was recorded when behavioral score decreased by at least two points for two consecutive days. Relapses were scored for an individual mouse when the behavioral score increased by at least two points for two consecutive days. Length and number of relapses were noted following peak disease throughout the 40-day treatment period.

Only those mice that survived the 40-day treatment period were included in behavioral assessments (RR-EAE+Saline, n=25; RR-EAE+LDN, n=22).

Neuropathology
Lumbar spinal cord tissues (L4-L6) were collected from mice after 5 (n=3-4/group), 14 (n=4-5/group), and 40 days of treatment. Mice were deeply anesthetized with a cocktail containing ketamine (30 mg/kg), xylazine (5 mg/kg), and acepromazine (2 mg/kg) diluted in sterile water and euthanized by intracardial perfusion with fresh 4% paraformaldehyde (PFA). Intact vertebral columns were dissected and post-fixed in 4% PFA for 18 hours; spinal cord tissue was coded in order to correlate behavioral scores and neuropathology. Tissues were processed for paraffin embedding or frozen for immunohistochemical staining following published protocols [18,19,23-27]. Controls for immunostaining included sections stained with secondary antibody only. At least 2 sections/animal from 3-8 animals/group were evaluated for each histopathological measure. The number of microglia and/or macrophages were measured by immunostaining with Iba-1 (1:200, Wako, Osaka, Japan) and/or F4/80 (1:200, ab6640, Abcam, Cambridge, MA) antibodies. Iba-1 only positive cells were identified in tissue on either side of the central canal; a positive cell had a DAPI-stained nucleus surrounded by rhodamine-labeled antibody (TRITC) with at least one visible projection. Macrophages were identified within the central ventral white matter and had DAPI-stained nuclei surrounded by both TRITC (Iba-1 positive) and FITC (F4/80 positive) labeling. Cell proliferation was determined using the cell cycle marker Ki67 (1:200, ab66155, Abcam). Astrocyte activation was measured using glial fibrillary acidic protein (GFAP) antibody (1:500, Dako, Carpinteria, CA); dual labeling of Ki67 and GFAP (1:300, Cell Signaling, Danvers, MA) antibodies enabled assessment of proliferating, activated astrocytes. T lymphocytes were quantified as total CD3-positive cells in each field [25,28] using a CD3 antibody (1:200, ab5690, Abcam). T lymphocytes, activated astrocytes, and proliferative cells were counted in the central ventral white matter at 20x magnification using Slidebook software (Intelligent Imaging Innovations, Ringley, CT). Percent Ki67+ cells were calculated by counting the number of DAPI-labeled cells and dividing by the number of Ki67-labeled cells. To assess astrocyte...
proliferation, GFAP+ and GFAP+Ki67+ cells were counted and the percentage of cells actively in the cell cycle were calculated.

Demyelination was evaluated in Luxol fast blue stained tissue (Roboz Surgical Instrument Co., Washington, DC) counterstained with 1% neutral red (Fisher Scientific, Pittsburgh, PA). Percentage of demyelination within cross sections of lumbar spinal cord (L4-L6) were determined by measuring total white matter area and dividing by demyelinated area, using Image-Pro 6.2 software (MediaCybernetics, Bethesda, MD) [29]. Values are expressed as mean ± standard error of mean (SEM). Images were captured using an Olympus IX-81 epifluorescent microscope at 20X magnification.

Statistical analysis
Non-parametric behavioral data were analyzed using Mann-Whitney U tests. Morphological data were analyzed using the two-tailed Students t-tests or one-way analysis of variance (ANOVA) and Newman–Keuls for subsequent comparison (GraphPad Prism, La Jolla, CA). P values less than 0.05 were considered statistically significant.

Results
Behavioral assessment
All SJL/J mice inoculated with PLP139-151 developed clinical signs of disease within 11 days. After 2 days of a clinical score of 0.5 or greater, mice were randomized into groups receiving i.p. injections of LDN (0.1 mg/kg naltrexone; n=22) or saline (n=25). The average day of disease onset occurred 9-10 days after immunization (Figure 1A). The latency time (days) to peak disease following initiation of treatment ranged between 3 and 4 days. No differences were noted in time of peak disease between LDN or saline-treated mice. The effects of LDN treatment on
behavior are presented in Figure 1A and 1B. Overall analyses of mice receiving LDN therapy indicated reduced behavioral scores throughout the 40-day observation period. Post-hoc data analyses of cumulative behavioral scores for individual mice revealed a dichotomous response to LDN. A portion of mice receiving LDN did not respond to treatment to the extent that other mice receiving LDN displayed (Figure 1B), thus establishing a biphase response to LDN of mice considered LDN-non-responders (LDN-NR) and LDN responders (LDN-R). "Responders" were considered those mice with an average behavioral score<3 over the 40-day treatment period. The behavioral profiles of these animals are presented in Figure 1B and indicate that within 2 days of LDN treatment, mean behavioral scores were significantly reduced from saline controls and non-responders. Mean behavioral scores were significantly lower than controls on 34 of the 40 days of treatment. In comparison to saline-treated mice that had a mean of 7.7 days of mild disease activity, RR-EAE+LDN animals had behavioral scores that were considered mild for approximately twice the time (13.4 ± 2.4 days).

Relapses were scored when a mouse displayed a daily behavioral score increase by at least 2 points for 2 consecutive days (Figure 2C). Eighteen saline-treated mice had relapses that extended for 14.1 ± 2.1 days, whereas LDN mice had relapses lasting 10.1 ± 2.1 days.

Neuropathology

Suppression of the immune response following LDN therapy

Proliferation of microglia/macrophages and T-lymphocytes is an indicator of the immunological response. To assess whether LDN diminished this early phase of autoimmunity, lumbar spinal cord sections were collected after 5 (acute disease), 14 (first relapse) and 40 (chronic disease) days of treatment and stained with antibodies to Iba-1 to measure the number of microglia/macrophages (Figure 3A and C), or CD3 to assess the number of CD3+ T lymphocytes (Figure 3B and D). Relative to non-immunized normal animals, the numbers of Iba-1 positive cells were increased in RR-EAE mice at all time points with no temporal pattern being expressed. Within 5 days of established

![Figure 2](image-url).

Figure 2. LDN therapy increased length of remission and decreased relapse time of mice with RR-EAE. Mice (responders and non-responders are combined) were treated with LDN beginning two days after the first signs of clinical disease. (A) Length of time (days) spent in complete remission (behavioral scores ≤ 0.5) during the 40 day treatment period. The length of time in complete remission was 11-fold longer (p<0.03) for mice responding to LDN. The average remission for saline-treated EAE mice was 0.4 ± 0.2 days, in comparison to 4.6 ± 2.16 days for LDN treated RR-EAE mice (Figure 2A).

In addition to periods of complete remission, mice treated with LDN displayed intervals of "mild" disease activity, defined as days with behavioral scores less than or equal to 2 (Figure 2B). In comparison to saline-treated mice that had a mean of 7.7 days of mild disease activity, RR-EAE+LDN animals had behavioral scores that were considered mild for approximately twice the time (13.4 ± 2.4 days).
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Disease, the average numbers of microglia/macrophages per field were approximately 82 in saline-treated mice and 16 in normal, non-immunized mice. LDN treatment significantly reduced the number of microglia/macrophages by 54 percent. At peak disease (day 14), RR-EAE+Saline mice had an average of 62 Iba-1+ cells per section in comparison to 6 microglia/macrophage positive cells in normals, and 45 Iba-1+ cells in RR-EAE+LDN mice, a 27% decrease.

T lymphocyte infiltration as detected by CD3+ cells was not observed in lumbar spinal cords on day 5 (Figure 3D). However, within 2 weeks of established disease, mice in the RR-EAE+saline group had approximately 114 lymphocytes per field in comparison to 6 CD3+ cells in LDN treated mice, and 26 CD3+ cells for normal mice. LDN treatment reduced the number of T-lymphocytes by 46% (p<0.01). On day 40 of LDN therapy, the number of CD3+ cells was reduced 52% by LDN in comparison to saline-treated RR-EAE mice (Figure 3D).

To distinguish between microglia and macrophages, double labeling with Iba-1 and F4/80 was conducted. F4/80 and Iba-1 positive cells were considered macrophages [30]. At 14 days of treatment, RR-EAE mice had more than 13-fold increase in macrophages in the central ventral white matter. LDN therapy for 2 weeks reduced macrophage infiltration by 50% (Figure 4).

LDN inhibits cellular proliferation and activation of astrocytes

Spinal cord sections stained with Ki67 provided an indicator of total cellular proliferation in RR-EAE and normal mice at 14 (Figure 5A) and 40 days of treatment. Evaluation of the number of Ki67+ cells in the central ventral white matter of the lumbar spinal cord at 14 and 40 days of treatment is presented in Figure 5C. At peak disease (day 14), saline-treated RR-EAE mice had approximately 9% proliferating cells per field, a 29% increase over normal mice. LDN therapy reduced cellular proliferation by approximately 74%. After 40 days of treatment, cell proliferation was substantially diminished in the spinal cord of all

Figure 3. LDN treatment repressed expression of microglia/macrophages (A,C) and T lymphocytes (B,D) in lumbar spinal cord from mice with established RR-EAE. Mice were evaluated at 5, 14, and 40 days of treatment. Lumbar spinal cord sections were stained with anti-Iba-1 to detect microglia/macrophages (A) or anti-CD3 to detect T lymphocytes (B); sections stained with secondary antibody only are shown in the inset. Bar = 50 μm. (C) The number of Iba-1+ cells visualized on either side of the central canal after 5, 14, and 40 days of treatment. (D) The number of CD3+ cells recorded in the central ventral white matter of spinal cord collected from mice treated with LDN (equal numbers of responders and non-responders) or saline for 14 or 40 days. Values represent means ±SEM for 3-6 animals per group. Significantly different from normal mice at p<0.05 (*), p<0.01 (**) and p<0.001 (+++); significantly different from RR-EAE mice receiving saline at p<0.05 (*) and p< 0.01 (**).
groups relative to day 14 (approximately 25 days after immunization). Nonetheless, LDN treatment of RR-EAE mice resulted in less than 1% cell proliferation in comparison to approximately 3% in the RR-EAE+Saline group, a 78% inhibition in cell proliferation following LDN therapy.

Activation of astrocytes using the marker GFAP (Figure 5B) demonstrated significant increases in the number of GFAP+ astrocytes at 5, 14, and 40 days of treatment (Figure 5D). RR-EAE+LDN mice had reductions in activated astrocytes at all time points monitored. Within 5 days of established disease and treatment, astrocyte activation was elevated 120% in the RR-EAE+Saline group relative to normal mice. LDN reduced this activation by 41% on day 5 and reduced the 5 fold activation of astrocytes on day 14 and day 40 by approximately 47%.

Proliferation of activated astrocytes was measured by double-labeling spinal cord sections and counting GFAP+ and Ki67+ cells in the central ventral white matter (Figure 5E and F). On day 14, saline-treated mice had 10.1 ± 2.0 cells per field in comparison to 1.5 ± 0.3 cells for RR-EAE+LDN mice, a reduction of 84% (p<0.001). At 40 days, the number of proliferating activated astrocytes in LDN-treated tissues was approximately 50% of that in spinal cords from saline-treated RR-EAE mice, but the differences did not reach statistical significance (Figure 5F).

LDN protects against demyelination in established RR-EAE

Demyelination was evaluated in spinal cord tissue after 14 and 40 days of treatment by staining with Luxol fast blue and neutral red (Figure 6A). Normal mice demonstrated negligible demyelination over the 40-day observation period. After 2 weeks of treatment, saline treated mice had approximately 22% demyelination in comparison to less than 5% (p<0.01) for RR-EAE+LDN animals (Figure 6B). By day 40, levels of demyelination were comparable between LDN and saline-treated mice.

Discussion

Multiple sclerosis frequently begins with the relapse-remitting form that presents with a wide spectrum of behavioral signs and progresses onto a more chronic progressive form [1,2,5]. This study demonstrates for the first time that alterations of the OGF-OGFr axis by upregulating endogenous opioids and receptors following a short duration of receptor blockade can reverse the pattern of relapses and promote a
sustained period of remission in mice with established RR-EAE. LDN treatment was effective at diminishing overall clinical behavior, and increasing the number and duration of remissions.

Unlike results recorded in mice when treatment of RR-EAE begins at the time of immunization, LDN did not markedly reduce the severity of the initial flair as it occurred within 2-4 days following initiation of therapy. However, if the mice responded to treatment, the reversal of the course of disease was detectable within 2-3 days of therapy.

The present study confirmed the role of the OGF-OGFr axis in EAE, and presumably MS, by demonstrating that LDN therapy, which indirectly...
upregulates production of OGF and OGFr, and represses relapses. The expanded behavioral scale allowed for detailed observations of motor and sensory skills in mice, and supported the distinct characteristics of either remission or relapse. The biphasic response of mice treated with LDN was identified over the course of several studies utilizing multiple observers, with at least one masked to treatment. Previous reports on mice immunized with PLP139-151 revealed comparable patterns of behavior among the saline-treated RR-EAE groups [19].

With regard to the pathobiology of the spinal cord, PLP139-151 immunization resulted in elevated expression of microglia and macrophages in lumbar spinal cord tissue, as well as increased activated astrocytes. Whether these accounts are a result of proliferation, migration, or a combination of both are unknown, although in previous tissue culture studies, OGF and naltrexone had no observable effect on cancer cell migration through matrix gel [31].

The therapeutic mechanism for both OGF and LDN involves an alteration of the OGF-OGFr axis with an influx of inhibitory peptide either directly (OGF injections) or indirectly by LDN feedback stimulation of peptide and receptor secretion and/or expression. In previous studies on RR-EAE when OGF therapy was initiated at the time of disease induction, mice showed markedly reduced clinical signs, significant reductions in relapses both in number and severity, and many mice with complete remission [18]. When OGF therapy was initiated after established disease, approximately 10 days after induction, mice responding to OGF had reduced clinical scores, longer remission periods, and fewer relapses [19]. Using the PLP139-151 induction model of SJL/J mice in this study also resulted in a group of mice that did not respond to treatment. Whether these biphasic responses are related to the genetic inconsistency of the SJL species [13] or whether there are other epigenetic factors involved, data collected from this animal model must be cautiously interpreted [32].

Although the mechanistic pathway for the amelioration of clinical disease following OGF or LDN treatment is not fully investigated in the RR-EAE model, the reduction in the number of T lymphocytes infiltrating spinal cord tissue, along with reductions in activated astrocytes, suggest that cell replication is inhibited. These observations support earlier work demonstrating that T cells activated in culture had repressed cell division following treatment with a variety of dosages of OGF or LDN [33]. The underlying evidence for inhibited cell replication is supported by our research on the chronic progressive model [23]. Investigations on the neuropathology of mice using the MOG-immunized model of progressive EAE showed that OGF suppressed cell replications of astrocytes, glia, and neurons, and moreover depleted nitric oxide synthesis of astrocytes [34].
The present study demonstrates that the OGF-OGFr axis is intricately involved with progression of RR-EAE, and presumably this translates to patients with relapse-remitting MS. Given that both OGF and now LDN, have been documented to change the course of disease in mice with EAE, it would suggest that the OGF is intact and able to interact with peptide or antagonist to subsequently inhibit proliferation. However, the levels of endogenous OGF may be insufficient to counter the inflammatory processes that follow immunization. Nonetheless, both OGF and LDN treatments are effective for progressive and relapse-remitting EAE supporting that a similar pathophysiology is involved in both disorders.

The present data support and extend our ongoing preclinical studies demonstrating the efficacy of LDN treatment in animal models of relapse-remitting EAE. Clinical trials using OGF or LDN as therapy have reported improved quality of life in MS patients [20,21] and together, the data warrant further randomized, controlled clinical trials in order to gain approval for LDN, or direct therapy with OGF, as treatment for patients with relapse-remitting or progressive MS.

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