Serum [Met^5]-enkephalin levels are reduced in multiple sclerosis and restored by low-dose naltrexone

Michael D Ludwig, Ian S Zagon and Patricia J McLaughlin

Department of Neural & Behavioral Sciences, College of Medicine, Pennsylvania State University, PA 17033, USA
Corresponding author: Patricia J McLaughlin. Email: pxm9@psu.edu

Impact statement

This report presents human and animal data identifying a novel biomarker for the onset and progression of multiple sclerosis (MS). Humans diagnosed with MS have reduced serum levels of OGF (i.e. [Met^5]-enkephalin) relative to non-MS neurologic patients, and low-dose naltrexone (LDN) therapy restored their enkephalin levels. Serum OGF levels were reduced in mice immunized with MOG_{35-55} prior to any clinical behavioral sign of experimental autoimmune encephalomyelitis, and LDN therapy restored their serum OGF levels. β-endorphin concentrations were not altered by LDN in humans or mice. Thus, blood levels of OGF may serve as a new, selective biomarker for the progression of MS, as well as response to therapy.

Abstract

Low-dose naltrexone is a widely used off-label therapeutic prescribed for a variety of immune-related disorders. The mechanism underlying low-dose naltrexone's efficacy for fatigue, Crohn's disease, fibromyalgia, and multiple sclerosis is, in part, intermittent blockade of opioid receptors followed by upregulation of endogenous opioids. Short, intermittent blockade by naltrexone specifically blocks the opioid growth factor receptor resulting in biofeedback events that increase production of the endogenous opioid growth factor (OGF) (chemically termed [Met^5]-enkephalin) facilitating interactions between opioid growth factor and opioid growth factor receptor that ultimately, result in inhibited cell proliferation. Preclinical studies have reported that enkephalin levels are deficient in animal models of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Our hypothesis is that serum enkephalin levels are diminished in humans with multiple sclerosis and experimental autoimmune encephalomyelitis mice, and that change in serum opioid growth factor levels may serve as a reasonable candidate biomarker for the onset of experimental autoimmune encephalomyelitis and response to therapy. To address this, we designed a two-part study to measure endogenous opioids in multiple sclerosis patients, and to investigate the temporal pattern of decline in serum enkephalin concentrations in mice with chronic progressive experimental autoimmune encephalomyelitis and treated with low-dose naltrexone. For comparison, we investigated whether low-dose naltrexone exposure in normal mice also resulted in altered enkephalin levels. In both animal models, we monitored tactile and heat sensitivity, as well as differential white blood cell counts as indicators of inflammation. Serum [Met^5]-enkephalin levels were lower in humans with multiple sclerosis relative to non-multiple sclerosis patients, and low-dose naltrexone restored their levels. In experimental autoimmune encephalomyelitis mice, [Met^5]-enkephalin levels were depressed prior to the appearance of clinical disease, and were restored with low-dose naltrexone treatment. Low-dose naltrexone therapy had no effect on serum [Met^5]-enkephalin or β-endorphin in normal mice. Thus, [Met^5]-enkephalin (i.e. opioid growth factor) may be a reasonable candidate biomarker for multiple sclerosis, and may signal new pathways for treatment of autoimmune disorders.

Keywords: Low-dose naltrexone, opioid growth factor, experimental autoimmune encephalomyelitis, [Met^5]-enkephalin, β-endorphin, ELISA

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Introduction

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system impacting more than two million persons worldwide.[1,2] MS is usually diagnosed between the second and fifth decades of life and affects women two to three times more than men. The etiology of MS remains unknown, but it appears that MS is multifactorial, with genetics and environmental factors influencing onset.[3] Currently, there are several FDA-approved disease modifying therapies (DMT) for the treatment of MS, and in particular for relapsing–remitting MS (RRMS), which accounts for 85% of the MS population.[4] There are very few DMTs for the treatment of primary progressive MS (PPMS). The DMTs that are available currently are expensive, often costing up to thousands of dollars per month. Many of the current therapies also have side effects that may include progressive

multifocal leukoencephalopathy (PML).[1,2,5] PML has occurred in patients taking natalizumab, fingolimod, and dimethyl fumarate. Finally, in addition to the cost and side effects, the method of administration, injection, IV infusion, or oral pill for the DMTs limits patient compliance. These issues have driven patients towards alternative therapies such as low doses of naltrexone (LDN). Although LDN therapy is off-label, it has received widespread use for several autoimmune disorders including fibromyalgia, Crohn's, and MS.[6–9] Recently, several new clinical trials on LDN are have been initiated nationally and internationally.[10] Whether LDN treats symptomology or is disease-modifying is unknown. Naltrexone is a general opioid receptor antagonist, and is FDA-approved for treatment of addiction, alcoholism, and even weight loss. Preclinical research on the use of naltrexone has confirmed that it is the duration of opioid receptor blockade that confers the effect.[11] It has been demonstrated in both animal[12] and tissue culture[13] studies that administration of naltrexone in low dosages (i.e. 0.1 mg/kg, LDN) resulted in a blockade lasting 4 to 6 h a day, during which time there is an increase in the levels of endogenous opioids. Following the intermittent blockade, there is an opportunity for heightened interaction of the elevated levels of endogenous opioids, one of which is opioid growth factor (OGF), chemically called [Met5]-enkephalin. OGF serves as an inhibitor of cell proliferation by upregulating the cyclin-dependent inhibitory kinases p16 and p21 that halt the cell cycle.[14] The selective receptor for OGF is a nuclear-associated, non-classic opioid receptor, sharing no structural similarities with the classical mu, delta, or kappa opioid receptors, and has been named OGFr.[15,16] OGFr is highly conserved across species and has been found in numerous cell types, including cancer cell lines.[15] The OGF-OGFr axis is a key regulatory pathway in maintaining homeostasis of immune cell replication.[17–19]

Modulation of the OGF-OGFr axis is effective in the treatment of experimental autoimmune encephalomyelitis (EAE), the accepted mouse model of MS. In both the chronic (Ch-EAE) and relapsing remitting (RR-EAE) models, treatment with either exogenous OGF or upregulation of endogenous enkephalins with LDN, effectively reduced the overall disease severity of EAE and diminished associated neuropathology.[20–24] In earlier studies using the chronic progressive model of EAE and treating immunized mice concurrently with LDN, data revealed that while all mice immunized with MOG and saline-treated expressed clinical behavioral signs by day 22, only 68% of the MOG + LDN mice ever presented with behavioral symptoms of EAE. Moreover, the maximum disease incidence for the MOG + LDN was reached on day 30 in comparison to day 22 for saline-treated mice. In addition to delays in disease onset, the maximal severity scores for mice receiving LDN were 37% less than that for vehicle- (saline) treated mice receiving MOG.[21] In studies whereby mice were immunized with PLP to induce relapsing–remitting EAE, LDN treatment initiated at the time of visible, established disease, LDN treatment significantly reduced behavioral scores across the 40 day observation period, increased the length of remission; spinal cord pathology from LDN-treated mice revealed reductions in the number of inflammatory cells (microglia/macrophages) and activated astrocytes, as well as decreases in areas of demyelination relative to saline-treated mice with RR-EAE.[24]

Some clinical trials have shown the safety of LDN,[6,7,25] and several chart reviews of patients administered LDN as an off-label supplementary treatment to FDA-approved DMTs, have reported favorable outcome.[26,27] Despite its lack of FDA approval, LDN therapy, even as a stand-alone therapy, remains popular. Patients on LDN have self-reported that LDN has had a positive impact on the quality of life and fatigue levels.[26,27]

Based on a comprehensive knowledge about the role of OGF (i.e. [Met5]-enkephalin), it was hypothesized that serum enkephalin levels are diminished in mice with EAE and humans with MS. Moreover, it is plausible that changes in serum OGF levels may serve as a biomarker for the onset of EAE disease and response to therapy. In this two-part study, we obtained human serum samples from a small number of patients diagnosed with MS and taking either Copaxone and/or LDN and measured [Met5]-enkephalin and β-endorphin levels. Copaxone or glatiramer acetate is small four amino acid polymer that serves as an immunomodulating drug. Although the mechanism is not well defined, Copaxone reduces the frequency of relapses in RRMS, but had little effect on progression of disease; the process may involve modulating the proportion of Th1 and Th2 regulatory T cells during inflammation. Chart reviews have supported evidence that the combination of Copaxone and LDN is not adversive.[26,27] In the second set of experiments, we established a mouse model of chronic EAE to assess fluctuations in [Met5]-enkephalin immediately following immunization, prior to disease onset, as well as periodically throughout peak disease in animals randomized to receive saline or LDN. In an effort to quantitate changes in serum levels of [Met5]-enkephalin during receptor blockade, blood was collected from Ch-EAE mice in the morning during a period of receptor blockade (when levels are expected to be high), and in the afternoon (when serum levels should be lower because the [Met5]-enkephalin is now interacting on tissue receptors). Finally, to evaluate LDN and serum [Met5]-enkephalin levels in non-diseased states, we examined normal mice treated with LDN for two weeks followed by a washout period of seven days. In both Ch-EAE and normal animals, we monitored tactile and heat sensitivity, as well as differential white blood cell counts to determine whether LDN affected parameters of sensitivity and inflammation. [Met5]-enkephalin (or OGF) levels change quickly following immunization and remain deficient throughout the disease. Treatment with LDN was beneficial. In this small sample of human tissue, as well as our mouse studies, data support that [Met5]-enkephalin (i.e. OGF) levels are a valid biomarker for progression of disease and response to therapy.

Materials and methods

Animals and immunization

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

prior to immunization. Animals were group housed in rooms maintained at $21 \pm 0.5^{\circ}C$ with a relative humidity of $50 \pm 10\%$ under standard conditions; food and water were available ad libitum. Two paradigms of animals were established – normal mice that received no injections and those that were immunized to induce chronic experimental autoimmune encephalomyelitis (Ch-EAE).[21] Ch-EAE was initiated by injection of 400 μg of myelin oligodendrocyte glycoprotein, residues 35–55 (MOG_{35-55}), dissolved in 0.2 ml of sterile phosphate-buffered saline (PBS) and emulsified with 0.15 ml incomplete Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO), which was completed with 750 μg mycobacterium tuberculosis(H37RA, Difco Laboratories, Detroit, MI).[22] The MOG and complete Freund's Adjuvant (CFA) emulsion was administered using subcutaneous injections to alternate flanks on days 0 and 7. On days 0 and 2, mice were given intraperitoneal (IP) injections of 500 ng pertussis toxin (PTX). A control subset of mice was injected with an emulsion of CFA and PBS in place of the MOG emulsion on days 0 and 7, and pertussis injections of days 0 and 2. These CFA + PTX mice served as ''controls.'' Non-immunized mice were considered ''normal.''

Animal treatment with LDN

Beginning at the time of disease induction, Ch-EAE mice and normal C57Bl6/J mice were randomly assigned to treatment groups receiving daily IP injections of either 0.1 ml PBS or 0.1 mg/kg naltrexone (LDN) (Sigma-Aldrich, St. Louis, MO) at 0800 hr. Treatment with LDN continued for 14 days, at which time treatment was halted, and both C57Bl6 and Ch-EAE mice had a one-week wash-out period prior to being euthanized on day 21 (week 3). Animals from both groups were periodically euthanized at weeks 1, 2, or 3 in order to collect blood.

Sensitivity testing

Touch (Von Frey filaments) and temperature (hot plate) sensitivity was measured; tests were conducted by observers masked to treatment.[28,29] Mice were subjected to these tests prior to the onset of the disease (baseline) and at one, two, and three weeks of disease. To measure temperature sensation, mice were placed on a specialized behavioral hotplate (TechniLab Instruments, Pequannock NJ, USA) set to $55^{\circ}C$. Mice were individually placed on the surface of the hot plate and the time recorded electronically until the behavioral response (e.g., rearing, paw licking and stamping, or escape) was observed. Mice were removed after 30 s if no response was observed. Following the first week of treatment with LDN, all testing was conducted at 1600 h. During the first week, testing was conducted at approximately 1100 h and 1600 h in order to determine the effect of the intermittent blockade of LDN.

For the Von Frey hair sensitivity testing, the mice were placed on a wire cage and lightly restrained. Von Frey filaments of increasing thickness were pressed against the hindlimbs of the mice until a response of foot withdrawal was observed. Each mouse received two trials and the force required to elicit the response was recorded and averaged for each mouse.

Murine white blood cell and differential cell counts

Blood was collected at baseline and during week one of LDN treatment from euthanized mice. Blood smears were prepared on autoclaved slides and stained using Wrights' stain (Easy III Quik Diff Stain, Azer Scientific). Total white blood cells were determined at 10× magnification; differential counts were assessed from a population of 100 cells at 100× magnification under oil immersion.

Murine serum collection

At time points throughout the study, mice were euthanized with CO_2 asphyxiation, and blood was collected through terminal cardiac puncture. Approximately 5 μL of whole blood was pooled on a slide to make a blood smear. The remaining blood volume was centrifuged at $4^{\circ}C$ at 12,000 r/min and stored at $-20^{\circ}C$ until assayed. After the seven-day time point, all blood was collected after 1600 h. For the seven-day time point, blood was collected from individual LDN mice at either 1200 h or 1600 h in order to determine the effect of the intermittent blockade following LDN on the serum enkephalin levels.

Human serum collection

Human serum samples were provided from the Institute for Personalized Medicine at the Pennsylvania State University College of Medicine in Hershey, PA. All samples were de-identified and assigned sample ID numbers by the Institute for Personalized Medicine prior to the start of the study. Samples were collected from volunteer MS patients receiving LDN therapy, either alone or in conjunction with glatiramer acetate (Copaxone), or Copaxone alone. Samples collected from patients who were not diagnosed with MS or other similar neurological conditions were considered controls.

ELISA assay for OGF and β-endorphin

Serum OGF levels were measured in both human and mouse blood samples using a solid phase Met Enkephalin ELISA kit (cat # MBS756178, MyBiosource; San Diego, CA). Sensitivity to both human and mouse was 1.0 pg/ml with no documented cross-reactivity between [Met^5]-enkephalin and other enkephalin analogues. Serum levels of β-endorphin were measured in human and mouse samples using the β-endorphin My Biosource ELISA kits specifically for human (cat #MBS2885899) and mouse (cat# MBS2881292); the detection range was 15–1000 pg/ml and sensitivity was < 6.74 pg/ml. ELISA plates were read on a BioTech Epoch plate reader at 450 nm; standard curve and concentration data were produced using a Gen5 2.0 Microplate Reader Software (Biotek).

Statistical analysis

All statistical analyses were performed with Graphpad Prism 5.0. Statistical tests performed included analysis of

variance (ANOVA), with Newman-Kuels posttests and two-tailed *t*-tests where appropriate.

Results

MS patient demographics

Serum from MS patients, as well as the non-MS controls, was collected during scheduled visits to the Pennsylvania State University Medical Center Neurology clinic (Table 1). The control population of non-MS patients was composed of two males and eight females, between the ages of 20 and 89. These individuals had no diagnosis similar to MS, but were visiting the clinic for other neurological issues (e.g. migraine headaches). The MS and Copaxone group was composed of three males and seven females, initially diagnosed with RRMS; one individual transitioned from RRMS to secondary progressive MS (SPMS) during the course of treatment. Individuals in this group had disease diagnosis for 4 to 37 years, and had been treated with Copaxone for 4 to 12 years. The second group of MS patients received both Copaxone and LDN at the time of blood sampling. This group was composed of three males and two females with a diagnosis of RRMS; one male was diagnosed with PPMS. These patients had MS for 3 to 43 years and were receiving Copaxone for three to nine years and LDN for less than eight years. A small cohort of MS patients was identified who were receiving only LDN at the time of blood collection. This group comprised two males and two females, with three patients diagnosed with RRMS and one patient who did not disclose the type of MS. Patients in this cohort had been diagnosed with MS for 4 to 23 years, and had been prescribed LDN for four to seven years. Information on the length of confirmed MS, as well as the length of time on either Copaxone or LDN, was provided by the patient, and not documented by medical records.

MS patient serum [Met^5]-enkephalin and β-endorphin concentrations

Human serum samples were analyzed using five ELISA kits for [Met^5]-enkephalin and one human β-endorphin kit. Serum [Met^5]-enkephalin levels for all MS and non-MS patients are presented in Figure 1(a). The non-MS control samples had a range of 21.6 to 53.9 pg/ml, with a mean of 37.0 ± 2.8 pg/ml. Mean serum levels for MS patients prescribed Copaxone only were 22.2 ± 2.3 pg/ml, a significant decrease of 40% from controls. MS patients prescribed both Copaxone and LDN had a mean serum enkephalin level of 20.5 ± 1.8 pg/ml, a decrease of 45% from controls. However, MS patients prescribed only LDN had serum enkephalin levels of 45.6 ± 5.9 pg/ml. Despite the relatively small sample size in this pilot study, OGF levels were elevated 2-fold (105%) above the level for a MS patient on Copaxone alone, and were greater than those recorded for non-MS patients by 23%.

As noted by the scattergram, there are individual differences in each group, most likely reflecting the severity and length of disease. Of special note was one patient in the MS and Copaxone group who was diagnosed with RRMS for 37 years, and had been on Copaxone for 12 years; this patient had a serum enkephalin level of 22.3 pg/ml. In the Copaxone and LDN group, one patient had been diagnosed with PPMS for approximately 43 years, the longest of any of

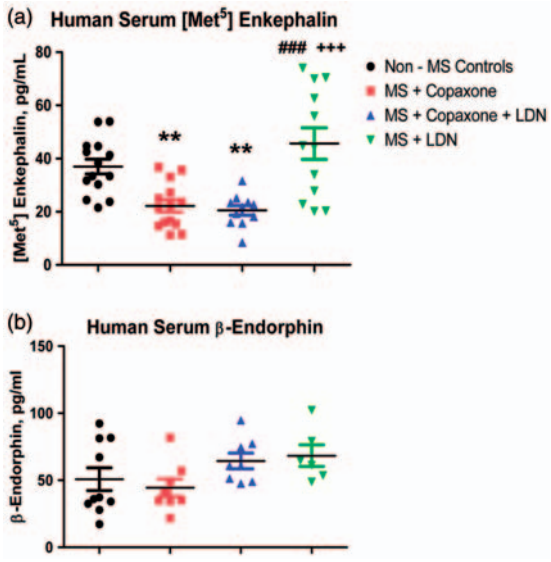

Figure 1 Human serum [Met^5]-enkephalin and β-endorphin concentrations recorded in volunteers from the Penn State Hershey Neurology Clinic. Subjects were diagnosed with MS and receiving Copaxone, Copaxone plus LDN, or LDN only; non-MS patients served as controls. Scattergrams represent individual patient values from two to three assays; each sample was assayed in duplicate. Significantly different from non-MS Controls at $P < 0.01$ (**); significantly different from MS + Copaxone at $P < 0.001$ (###) and from MS + Copaxone + LDN at $P < 0.001$ (+++). (A color version of this figure is available in the online journal.)

Table 1 Demographics of human subjects at the Penn state Hershey neurology clinic

	Age yrs	Gender M/F	Diagnosis	Length of MS diagnosis	Length copaxone treatment	Length LDN treatment
Non-MS controls	20–89	2/8	Not MS	————————	————————	————————
MS + Copaxone	38–68	3/7	9 RRMS, 1 SPMS	4–37 years	4–12 years	————————
MS + copaxone + LDN	36–73	3/2	4 RRMS, 1 PPMS	3–43 years	3–9 years	4–8 years
MS + LDN	40-66	2/2	3 RRMS[a]	4–23 years	————————	4–7 years

Note: Demographic information patients providing blood samples. Data on length of MS diagnosis and number of years on Copaxone or LDN were supplied by the patient. In some cases, Copaxone may have been prescribed prior to a diagnosis of clinically definite MS.
[a]One patient did not disclose the type of MS.
MS: multiple sclerosis; RRMS: relapsing–remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis; LDN: low-dose naltrexone.

the sampled patients. Not only is this a different subtype of MS from the remainder of the patients sampled, but also this patient had been diagnosed for a substantially longer period of time than all other patients. This patient had a mean serum enkephalin level of 12.2 pg/ml. For those taking LDN alone, most had previously been on disease-modifying therapies and chose to cease treatment. One patient had MS for 23 years, and had a serum enkephalin level of 32.6 pg/ml. The second patient had been recently diagnosed with RRMS within the last three years; however the length of LDN therapy was not disclosed. This patient had a serum enkephalin level of 39.2 pg/ml.

Serum β-endorphin concentrations were recorded as 50.8 ± 8.5 pg/ml for non-MS patients and approximately 25% higher in MS patients taking LDN (Figure 1(b)). Although MS patients not taking LDN had lower mean endorphin values, no statistical significance was noted between any group.

Serum samples were also subjected to an ELISA for C-reactive protein (MyBiosource, San Diego, CA). Measurements were inconclusive with wide ranges for both non-MS and MS patients most likely reflecting differences in the time of day serum was collected and the overall general health of patients at the time of serum collection.

Ch-EAE mouse serum [Met^5]-enkephalin concentrations

Serum samples were collected from 71 Ch-EAE mice, 5 CFA + PTX mice, and 49 normal mice. Five separate experiments with immunized mice were conducted. Serum was analyzed using four individual ELISA kits for [Met^5]-enkephalin and two ELISA kits for β-endorphin. All mice immunized with MOG_{35-55} presented with disease by day 10. In these experiments, several groups of mice were euthanized prior to the onset of clinical disease at seven days after initiation of MOG_{35-55} injections. A primary objective of this study was to determine if changes in serum enkephalin (i.e. OGF) levels occurred during the first seven days and preceded behavioral signs of clinical disease in the EAE model (Figure 2(a)). Blood samples collected from control mice receiving CFA + PTX were shown to have a mean (±SEM) serum enkephalin concentration of 41.9 ± 7.3 pg/ml, which was not significantly different from the normal untreated controls of 48.6 ± 4.9 pg/ml. Comparison of the CFA + PTX mice and Ch-EAE mice treated with either saline or LDN is presented in Figure 2(a). Within one week of immunization, and prior to confirmation of clinical disease, Ch-EAE mice treated with saline had significant reductions (51%) in serum enkephalin levels over CFA + PTX mice, and 58% reductions in serum enkephalin relative to normal control mice. After seven days of treatment with LDN, Ch-EAE mice receiving LDN in the morning were shown to have a significant elevation in serum OGF levels when blood was sampled in the morning and substantially less enkephalin in the serum when blood was sampled in the afternoon at 1600 h. Specifically, enkephalin concentrations in CH-EAE + LDN mice were 65.9 ± 7.4 pg/ml in the morning and 25.8 ± 7.5 pg/ml approximately 6–7 h later, at 1600 h. These fluctuations were expected as enkephalin leaves the blood stream readily and is either bound to receptors or degrades.

Ch-EAE mice continued to receive LDN therapy for a total of two weeks, followed by a one-week washout period that ended 21 days after immunization. At week 3 of the study corresponding to peak disease for this model of EAE, Ch-EAE mice had mean clinical scores of 2.25 and 1.1 for the saline group and LDN group, respectively. At week three (peak disease) Ch-EAE mice treated with saline had a mean serum OGF level of 16.9 pg/ml, which was significantly different from the normal, and a further reduction of 35% from the week one time point (Figure 2(b)). The LDN mice following the one-week washout from LDN was found to have a mean serum OGF level of 57.8 pg/ml, which was significant to the saline-treated mice at this time point. These data display a reduction in enkephalin levels with disease that can be reversed and maintained with LDN, even following the cessation of therapy.

Ch-EAE mouse serum β-endorphin concentrations

Serum endorphin levels were measured in CH-EAE treated with either saline or LDN for 7 or 14 days and then at 21 days following a 7-day washout period (Figure 2(c)). No differences in β-endorphin concentrations were noted between normal mice and those with Ch-EAE and treated with either saline or LDN. Moreover, no differences in endorphin levels were noted in blood from LDN-treated mice collected in the morning or afternoon.

Normal mice serum [Met^5]-enkephalin concentrations

Because the mechanism underlying the action of LDN is that the low dosages of naltrexone intermittently block opioid receptors resulting in a feedback loop of increased enkephalin production, followed by a period of time when the receptors are devoid of antagonists, and the enkephalins interact with excess receptors resulting in exuberant action (i.e. inhibitory action), it is necessary to measure enkephalin levels at two time points. Concentrations of enkephalins in the morning, within 3–4 h of LDN injection should theoretically demonstrate elevated serum levels. Measurements taken 8–10 h after LDN injection (i.e. 1600 or 1800 h), could reflect normal or lower levels suggesting that the enkephalins are interacting with receptors and not circulating in the blood.

It was important to study this phenomenon in normal mice receiving LDN in order to compare LDN treatment with EAE mice (Figure 3). Normal C57Bl6/J mice received daily saline or LDN injections at 0800 h for one week. Serum enkephalin concentrations were examined at 1200 and 1600 h (Figure 3(c)). In comparison to normal animals that had 48.6 pg/ml (range: 32.9 and 72.2 pg/ml) enkephalin concentrations prior to receiving either LDN or saline, C57BL/6J mice injected with LDN for seven days had a mean serum OGF level of 63.3 pg/ml, an increase of 30% over the baseline when blood samples were taken during receptor blockade. Following the LDN receptor blockade, the mice had a mean serum OGF level of 48.5 pg/ml. Blood samples collected after two or three weeks of treatment revealed mean serum enkephalin concentrations of

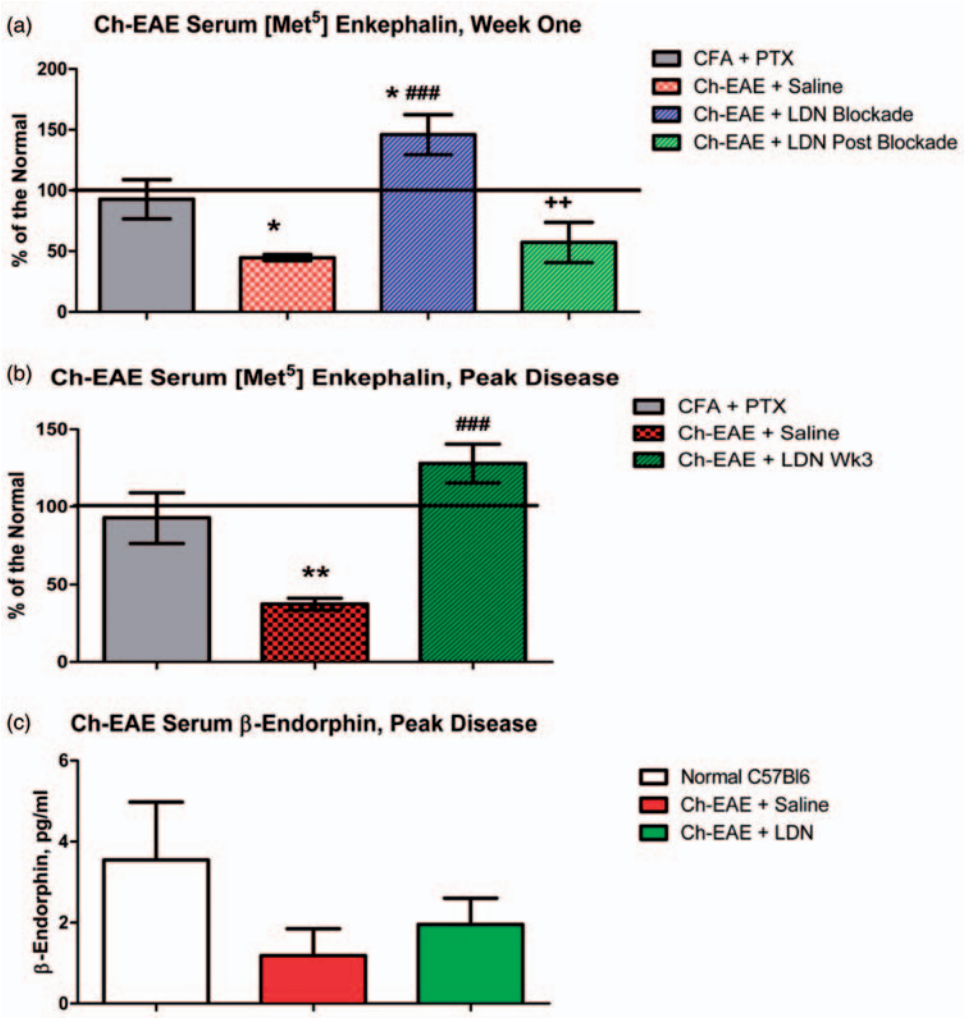

Figure 2 Serum [Met^5]-enkephalin and β-endorphin concentrations in mice immunized with MOG_{35-55} to induce Ch-EAE. Mice receiving only Complete Freund's adjuvant (CFA) and pertussis toxin (PTX) served as controls. Ch-EAE mice were randomized into groups receiving either sterile saline (Ch-EAE + Saline) or LDN (Ch-EAE + LDN). (a) [Met^5]-enkephalin concentrations in blood samples collected during receptor blockade (1000 hr) or following receptor blockade (1600 h) of seven days of LDN treatment. (b) [Met^5]-enkephalin concentrations in mice with Ch-EAE receiving either saline or LDN for three weeks (peak disease). (c) β-endorphin concentrations in mice with Ch-EAE receiving either saline or LDN for three weeks (peak disease). In panels A and B, values represent means ± SEM of the percent of normal, based on ELISA assays. Values (pg/ml) in panel C are means ± SEM based on two ELISA assays. Samples were assayed in duplicate for each ELISA. Significantly different from CFA + PTX at $P < 0.05$ (*) or $P < 0.01$ (**); significantly different from the Ch-EAE or Ch-EAE + Saline groups at $P < 0.001$ (###) and between the two time periods of LDN therapy in panel A at $P < 0.01$ (++). (A color version of this figure is available in the online journal.)

48.4 to 60.4 pg/ml. These data suggest that in healthy mice, LDN is unable to cause fluctuations in enkephalin levels that are detectable in the serum.

Total and differential white blood cell counts in mice with Ch-EAE and treated with LDN

Differential white blood cell counts can be an indicator of infection, inflammation, and/or other immune challenge. Mice immunized with MOG_{35-55} had elevated numbers of leukocytes (Figure 4), but those mice injected with LDN for seven days had significant reductions in total leukocytes relative to mice receiving saline. With regard to differential white blood cell counts, neutrophils were elevated in both Ch-EAE groups, whereas lymphocytes were decreased. Eosinophils, often an indication of infection or allergy, were comparable to normal values and significantly lower in the Ch-EAE + LDN group relative to those values in Ch-EAE mice receiving saline.

Ch-EAE sensitivity following LDN therapy

To assess the sensitivity to touch and heat in mice with Ch-EAE, mice receiving LDN or saline and immunized with MOG_{35-} were subjected to Von Frey Hairs tests on hind paws and hot plate tests (Figure 5(a)). In comparison to mice receiving CFA and PTX only that had a hot-plate response of 14 ± 1.5 s, Ch-EAE mice receiving saline for seven days had 9.3 ± 0.9 s indicating a heightened reaction to the hot plate and increased sensitivity. At one week, before any clinical signs of disease, Ch-EAE mice receiving LDN had hot plate responses comparable to control CFA + PTX mice, and significantly longer than Ch-EAE mice receiving saline; the hot plate responses were elevated

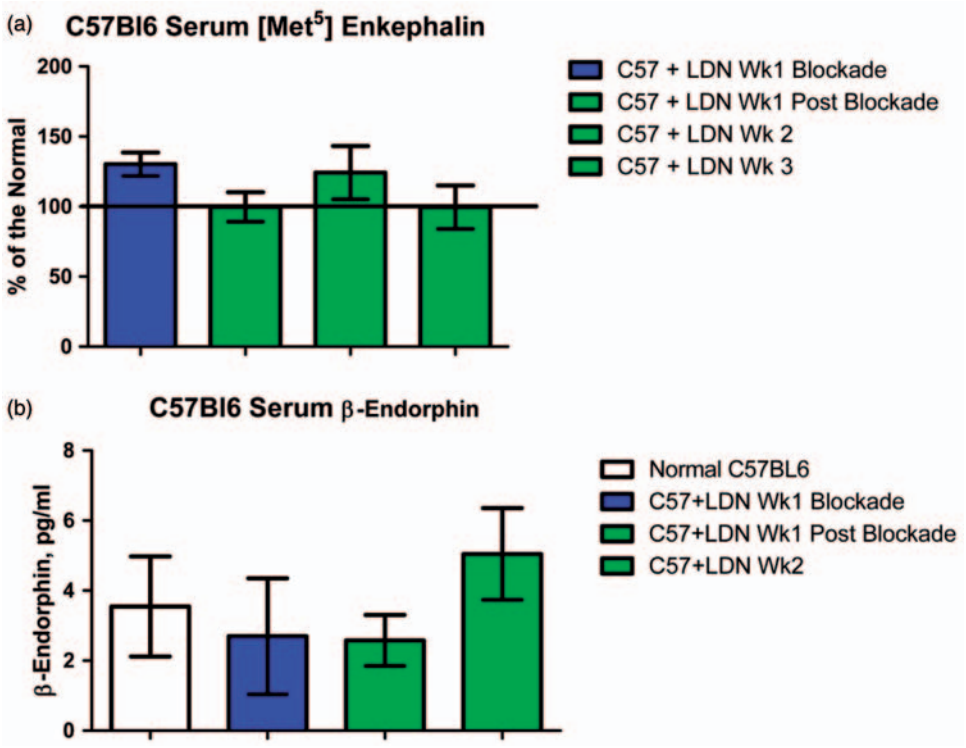

Figure 3 Serum [Met^5]-enkephalin and β-endorphin concentrations in normal, non-diseased mice. (a) Serum [Met^5]-enkephalin levels in normal mice receiving LDN for one or two weeks followed by a washout period for seven days (week 3). Blood samples were collected at seven days during receptor blockade (1000 h) and post blockade (1600 h). Values are means ± SEM of the percentage of normal mice receiving saline. (b) Serum β-endorphin concentrations for normal mice and those injected with LDN for one or two weeks. Blood samples were collected at seven days during receptor blockade (1000 h) and post receptor blockade (1600 h). Values are means ± SEM and expressed as percent of normal (a) or pg/ml (b). LDN exposure did not alter enkephalin or endorphin levels in normal mice. (A color version of this figure is available in the online journal.)

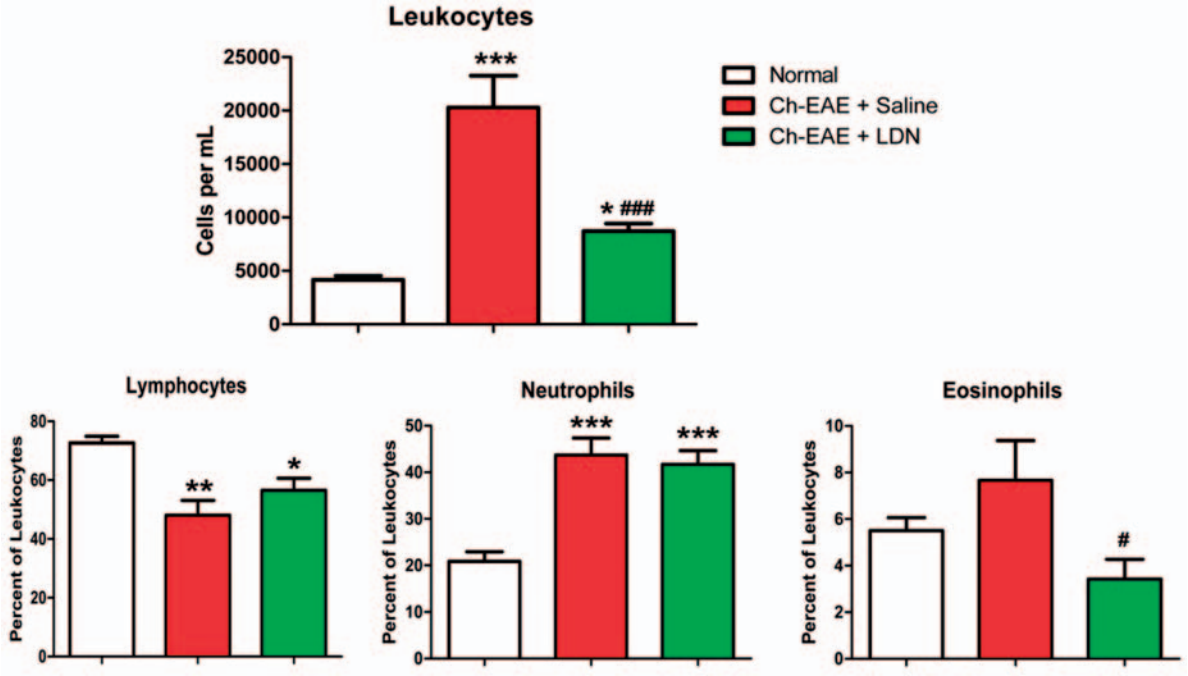

Figure 4 Total leukocyte and differential white blood cell counts were altered by EAE and modified by LDN. The top panel presents total number of leukocytes (per ml) in blood samples taken from normal mice or those with Ch-EAE and treated with saline or LDN for one week. The bottom panel presents differential cell counts of lymphocytes, Neutrophils, and eosinophils. Values represent means ± SEM for counts (100 cells at 100×) from three blood smears for each group, and are presented as total cells (leukocytes) or as the percentage of leukocytes. Significantly different from normal values at $P < 0.05$ (*), $P < 0.001$ (**), or $P < 0.001$ (***); significantly different from Ch-EAE + Saline group at $P < 0.05$ (#) or $P < 0.001$ (###). (A color version of this figure is available in the online journal.)

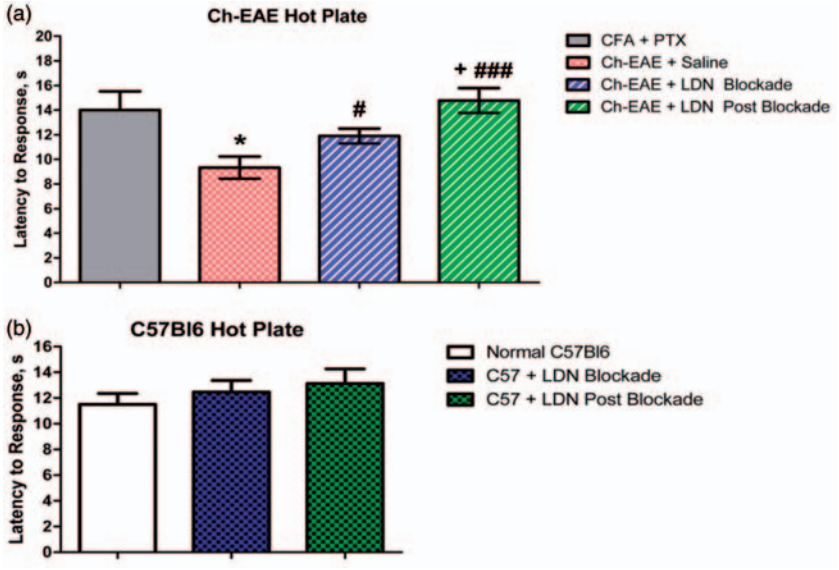

Figure 5 Sensitivity to heat, but not tactile stimulation was restored in EAE treated with OGF. Temperature sensitivity was measured using a hot plate (a) and tactile stimulation was measured using von Frey hairs (b) were measured during disease induction (days 0–5), disease onset (days 9–15), or peak disease (days 18–40). Treatment groups (n = at least 10) included non-immunized mice (Normal), mice immunized with MOG_{35-55} but not yet expressing EAE behavior (Ch-EAE), and mice with established disease and treated either with 10 mg/kg OGF (Ch-EAE + OGF) or saline (Ch-EAE + Saline) beginning at the time of established disease. Histograms represent means ± SEM; data from multiple observations of a single behavioral test and recorded within a timeframe (e.g. disease induction) were averaged. Significantly different from normal values at $P < 0.001$ (***); significantly different from Ch-EAE + Saline group at $P < 0.001$ (###). (A color version of this figure is available in the online journal.)

during both the blockade by LDN (morning) and post-blockade periods (afternoon). Hot plate latencies in Ch-EAE mice receiving saline or LDN for two weeks, or at week 3 following the seven-day washout period, were all comparable to baseline.

With regard to sensitivity to touch stimuli, all mice immunized with MOG_{35-55} had significantly heightened responses to Von Frey stimulation. All Ch-EAE mice responded to less than 0.05 g force in comparison to 0.15 g force required to elicit a withdrawal response at non-immunized mice or 0.1 g force required for a response in mice receiving CFA + PTX. No differences in responses were noted between LDN or saline treatments over the three-week period of testing (data not shown).

Normal C57Bl6 sensitivity following LDN

To assess any changes in sensitivity caused by LDN in normal mice, animals were subjected to hot plate testing at the same time points as the serum collection (Figure 5(b)). LDN had no effect on hot plate responses in normal mice. Response times ranged between 11.5 and 13 s for baseline and measurements taken after one or two weeks of LDN, or following a one-week washout period at week 3. Likewise, LDN had no effect on Von Frey hair stimulation in normal mice.

Discussion

Data from these experiments demonstrate two new concepts related to the pathophysiology of MS. These studies are the first to demonstrate quantifiable reductions in concentrations of [Met^5]-enkephalin (i.e. OGF) in the serum of patients with MS relative to non-MS patients. Furthermore, in our small sample of MS subjects, patients on LDN therapy alone or combined with Copaxone had higher serum OGF levels than MS patients not taking LDN. The data are based on random subjects, few in number, but nonetheless showed significant differences even for treatment with LDN alone. Interestingly, the endogenous opioid β-endorphin, often considered the ''feel-good'' peptide secreted during periods of exercise, was comparable in all non-MS and MS patients whether they were taking LDN or not. These data suggest that not all endogenous peptides are altered by LDN, and that perhaps OGF is more selective for MS and systemic responses to therapy. Further studies with more controlled periods of disease and treatment are needed to validate this initial observation. However, the diminished levels of the inhibitory peptide OGF may contribute to the robust inflammatory reaction. Nonetheless, the human data support our hypothesis that depressed enkephalins contribute to the initial autoimmune reaction and possible periodic flares. The onset of these events may in part be due to depressed enkephalins that are unable to suppress the robust proliferation of T and B cells, and resultant release of their cytokines that subsequently triggers the flare.[30] This study was also limited by the disease-modifying therapy used in conjunction with LDN. At the Hershey Neurology Clinic, Copaxone is frequently prescribed as the initial drug treatment upon first diagnosis. In an ideal clinical study, blood samples collected at the time of clinically isolated syndrome and followed through diagnosis of definite MS would support our observations on the role of [Met^5]-enkephalin in the disease process.

With regard to the animal model of EAE, these studies are the first to demonstrate changes in serum

concentrations of [Met^5]-enkephalin (i.e. OGF) prior to the onset of any clinical behavioral signs of the disease. Within seven days of initial immunization, simultaneous with the second injection of MOG_{35-55}, serum OGF concentrations were significantly reduced from baseline or from those recorded in normal mice, or in mice receiving CFA and PTX only, suggesting that the antibody reactions to MOG_{35-55} contributed to the decline in OGF levels. These data support our hypothesis that deficiencies in OGF levels contribute to the onset of autoimmune disorders. Most likely, the decreased levels of inhibitory peptides are unable to suppress the activated T and B cells, along with the heightened secretion of cytokines associated with immunological insult.[31] In a recent paper, it was demonstrated that mice immunized with MOG_{35-55} had significantly decreased OGF levels prior to the onset of disease,[31] and along with our data that OGF inhibits stimulated T and B cells,[17,18] suggests that reduction in the presence of functioning enkephalins may contribute to inflammation associated with autoimmune disorders. This theory is supported by our previous data in EAE mice who were treated with LDN or OGF therapy beginning at the time of immunization with either MOG_{35-55} or proteolipid $protein_{139-151}$ to induce progressive EAE or relapsing-remitting EAE, respectively.[20,21,23] In some cases, mice immunized with MOG and injected with OGF never developed clinical signs of the disease[20,21] The present study now documents the serum levels in Ch-EAE mice receiving LDN and shows that LDN treatment was able to restore enkephalin levels within seven days.

Finally, the biological basis of the mechanism underlying LDN activity was tested. It has been implicated from other studies that the duration of receptor blockade by LDN is important.[11] The intermittent blockade stimulates upregulation of enkephalins in the blood. Following blockade, the elevated enkephalins are either quickly degraded or may leave the circulation to interact with receptors on tissues and act to inhibit cell replication. This surge of enkephalins, but not β-endorphin, in the serum followed by an equilibrium of several hours post receptor blockade was documented in the Ch-EAE mice, but not normal mice.

Whether this down-regulation or dysregulation of enkephalins is part of the overall pathogenesis of MS or is a general phenomenon of autoimmune disorders is unknown and will require numerous animal model studies. However, in this imperfect model of MS, it was demonstrated that even prior to the onset of observable disease symptoms, there is a reduction of the endogenous enkephalins. What remains unanswered is why there is such a downregulation. Given that LDN was able to cause changes to the serum OGF levels in the normal mouse, in a diseased mouse, and in this small cohort of humans, both normal and diseased, suggests that manipulation of the OGF-OGFr axis is a fundamental regulatory pathway in the immune regulatory system. The changes in serum enkephalins are sufficient to consider serum OGF levels as a new and novel biomarker for onset and progression of MS, as well as response to therapy.

Authors' contributions: All authors participated in the design of the study, interpretation of data and/or review of the manuscript. MDL conducted the experiments, analyzed data, prepared the figures, and drafted the manuscript as part of his doctoral thesis research. ISZ and PJM contributed to the design of the overall research, provided interpretation of the data and prepared the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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