MODULATION OF THE OGF-OGFr AXIS ALTERS THE DISEASE COURSE OF RELAPSE REMITTING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

A Dissertation in

Anatomy

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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ABSTRACT

Multiple sclerosis is a chronic autoimmune disease of the central nervous system and affects over 2.3 million individuals worldwide. The etiology of MS is unknown. However, epidemiological evidence suggests a combination of environmental and genetic factors. No cure exists for MS. Current FDA-approved therapies target limited aspects of the disease, are expensive, and have undesirable side-effects. Experimental autoimmune encephalomyelitis (EAE) is the most widely used animal model for MS. EAE is a CD4+ T cell disease that can be induced in mice through immunization with a myelin antigen, such as proteolipid protein (PLP139-151) in EAE susceptible mice like the SJL/J-strain. Modulation of the opioid growth factor (OGF)-opioid growth factor receptor (OGFr) axis, by administration of OGF or low dose naltrexone (LDN), in both chronic and/or chronic established EAE diminishes disease severity and reduces the histopathological damage associated with the disease. This holds promise for those patients who experience the progressive form of the disease; however, 85 percent of MS patients experience the relapse-remitting disease course. It is unknown whether OGF and/or LDN will be an effective treatment option for RR-MS patients.

The central hypothesis is that modulation of the OGF-OGFr axis alters the course of relapse-remitting EAE (RR-EAE). The objectives of this research were to establish a working model of RR-EAE utilizing immunization of the SJL/J mouse with PLP139-151 and to assess the safety and efficacy of LDN or OGF when treatment is initiated at the time of induction or at the time of established clinical disease. Behavior and morphology of
glia, macrophages, and neurons were evaluated, as well as the effects of treatment regimens on CD4\(^+\) T lymphocyte infiltration into the CNS.

The first study established an animal model for relapse-remitting experimental autoimmune encephalomyelitis by immunization of SJL/J mice with PLP\(_{139-151}\). Treatment with OGF or saline was initiated simultaneously with immunization, and within 9 days, behavioral signs of RR-EAE were observed. OGF-treated RR-EAE animals had less severe clinical disease than mice receiving saline and exhibited 66% reductions in median cumulative disease scores, as well as prolonged periods of remission and diminished number and length of disease relapses. Neuropathological examination of lumbar spinal cord revealed reduced numbers of Iba-1 and CD3\(^+\) reactive cells, suggesting that OGF inhibited proliferation of microglia/macrophages and T lymphocytes, as well as decreasing the number of proliferating activated astrocytes (Ki67 and GFAP staining). Areas of demyelination and neuronal damage were markedly reduced after the 55-day observation period.

The second study examined the therapeutic efficacy of OGF in an established RR-EAE model. Two days following establishment of clinical disease, treatment with OGF or saline was initiated, and mice were observed on a daily basis. OGF treated mice had markedly reduced clinical signs of disease over the course of 40 days. OGF treatment increased the incidence and lengthened the time of remissions relative to saline-treated mice with RR-EAE. OGF therapy also reduced relapses, and facilitated extended periods of mild disease. Neuropathological examination of lumbar spinal cord after 40 days of treatment revealed decreased numbers of Iba-1 and CD3\(^+\) reactive
cells, suggesting that OGF inhibited proliferation of microglia/macrophages and T lymphocytes, as well as decreasing the number of proliferating activated astrocytes (Ki67 and GFAP dual labeled sections). Peptide treatment for 40 days diminished levels of demyelination in comparison to saline-treated mice with RR-EAE.

The third study examined modulation of the OGF–OGF receptor (OGFr) axis by low dose naltrexone (LDN) as a disease modifying therapy for established RR-EAE. After two days of clinical disease, mice received LDN or saline for 40 days. Mice were euthanized at study endpoints and spinal cords collected for neuropathological evaluation of glia, T lymphocyte infiltration, and demyelination. LDN treatment significantly reduced behavior scores across the 40 day observation period. The number of remissions was increased in LDN-treated groups relative to controls. A bimodal distribution of behavioral responses to LDN distinguished “responders” from mice considered “non-responders” that showed behavioral characteristics similar to saline-treated animals. More than 60% of the mice responding to LDN displayed several days of remission. LDN-treated mice also had reduced areas of demyelination and decreased numbers of macrophages/microglia and activated astrocytes, as well as reductions in spinal cord demyelination, relative to saline-treated controls.

The fourth study examined whether OGF or LDN alter Th effector responses of CD4$^+$ T lymphocytes within the CNS in established EAE. SJL/J mice were immunized with PLP$_{139-151}$ and treated with OGF, LDN or saline after the second consecutive day of clinical disease. After five treatment days, EAE mice were euthanized and brains and spinal cords collected for intracellular staining. Mononuclear cells were stained were
surface stained with anti-CD4, followed by intracellular and transcription factor staining with antibodies for IFNγ, IL-17, IL-4 and Foxp3 in order to assess the presence of CD4⁺ Th1, Th17, Th2, and Treg effector cells in the CNS. Flow cytometry analysis demonstrated that OGF and not LDN decreased CD4⁺ T lymphocytes present in the CNS of SJL/J mice with EAE at peak disease. However, modulation of the OGF-OGFr axis did not result in changes to CD4⁺ Th effector cell responses.

In conclusion, the data from these studies demonstrate an active role for endogenous opioids in autoimmune diseases such as MS and EAE. Furthermore, these results support previous research indicating that modulation of the OGF-OGFr axis, through either exogenous OGF or intermittent blockade with LDN, can be safe and effective treatment options for EAE and MS. Of particular interest were the opposing effects of OGF and LDN on CD4⁺ T lymphocytes present in the CNS of established EAE mice, and the lack of effect on different effector cell subpopulations. These results add to the knowledge previously elucidated from studies using the chronic-EAE model, and have opened the door to ongoing research to discern the mechanisms behind OGF and LDN in animal models of EAE, and ultimately the pursuit of clinical trials on OGF and/or LDN for treating MS.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>degrees Celsius</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CFA</td>
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<td>CIS</td>
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<tr>
<td>CSF</td>
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<td>3,3’-DAB</td>
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<td>DADLE</td>
<td>[D-Ala2-D-Leu5]-enkephalin</td>
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<td>dissemination in space</td>
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<tr>
<td>DIT</td>
<td>dissemination in time</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<td>disease modifying therapy</td>
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<td>DNA</td>
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<td>dipeptidyl peptidase IV</td>
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<td>experimental autoimmune encephalomyelitis</td>
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<td>EBV</td>
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<td>electrocardiogram</td>
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<td>GA</td>
<td>glatiramer acetate</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
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<tr>
<td>HCl</td>
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<tr>
<td>HDN</td>
<td>high-dose naltrexone</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>Iba-1</td>
<td>ionized calcium binding adaptor molecule 1</td>
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<td>interferon gamma</td>
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<td>immunoglobulin G</td>
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<td>interleukin</td>
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<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
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<tr>
<td>IQR</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<td>KOR</td>
<td>kappa opioid receptor</td>
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<td>low-dose naltrexone</td>
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<td>LFB</td>
<td>luxol fast blue</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>Definition</td>
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</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<td>min</td>
<td>minute</td>
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<td>MMP</td>
<td>metalloproteinases</td>
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<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
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<td>microliter</td>
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<td>micrometer</td>
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<tr>
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<td>major histocompatibility complex</td>
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<td>mu opioid receptor</td>
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<td>magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NAWM</td>
<td>normal appearing white matter</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NMS</td>
<td>normal mouse serum</td>
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<td>NMSS</td>
<td>National Multiple Sclerosis Society</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NTX</td>
<td>naltrexone</td>
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<tr>
<td>OGF</td>
<td>opioid growth factor</td>
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<td>OGFr</td>
<td>opioid growth factor receptor</td>
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PBS  phosphate-buffered saline
PFA  paraformaldehyde
PHA  phytohaemagglutinin
PLP  proteolipid protein
PMA  phorbol 12-myristate 13-acetate
PML  progressive multifocal leukoencephalopathy
POMC pro-opiomeranocortin
PPNK-d preproenkephalin-deficient
PP-MS primary progressive multiple sclerosis
PR-MS progressive relapsing multiple sclerosis
RBC  red blood cell
RR-EAE relapse remitting experimental autoimmune encephalomyelitis
RR-MS relapse remitting multiple sclerosis
RNA  ribonucleic acid
RPM  revolutions per minute
S phase synthesis phase
SEM  standard error of the mean
siRNA small interfering RNA
SJL/J  Swiss Jim Lambert/The Jackson Laboratory
SMI-32 neurofilament H non-phosphorylated
SPB  Sorenson’s phosphate buffer
SP-MS secondary progressive multiple sclerosis
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<td>T cell receptor</td>
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<tr>
<td>TGF</td>
<td>tumor growth factor</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>Treg</td>
<td>regulatory T lymphocyte</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethyl rhodamine iso-thiocyanate</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<td>WBC</td>
<td>white blood cell</td>
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CHAPTER 1. INTRODUCTION
1. Introduction

1.1 Multiple Sclerosis

1.1.1 Epidemiology and Etiology

Multiple sclerosis (MS) is a debilitating autoimmune disease of the central nervous system (CNS) affecting approximately 2.3 million individuals worldwide.\(^1\) In the United States, there are an estimated 10,400 newly diagnosed cases of MS per year.\(^2\) It is the most common CNS disease resulting in permanent disability in young and middle-aged individuals.\(^3,4\) The incidence of disease for females is 2-3 times higher than for males.\(^1,5-7\) The age of onset for MS ranges from 15-50, with the most common time of diagnosis occurring between 20-40 years of age.\(^5,6\) The incidence and prevalence of MS have increased globally, which may be due to better diagnostics, increases in reporting countries, or changes in survival rates.\(^1,5\) MS is more prevalent in countries that are temperate compared to those near the equator, and closer to the North and South poles. Thus, inhabitants of North America, Northwest Europe and the southern aspect of Australia have a higher risk of developing MS.\(^3-5\) There is an overall consensus among MS societies that these numbers underestimate the total prevalence of MS among the population, which may be attributed to the lack of reporting agencies and countries, as well as the lack of national registries.\(^4,6\)

The cause of MS remains unclear, however, genetic and environmental factors contribute to disease onset based on epidemiological studies. Familial-based studies have shown strongest ties for a genetic influence increasing the risk of developing the disease. First-degree relatives are typically 15-35 times more likely to develop MS.\(^3\)
Twin studies have shown that the risk among monozygotic twins is elevated compared to dizygotic twins.\textsuperscript{8} Siblings of those affected by MS have a level of risk related to the level of genetic commonality.\textsuperscript{8,9} That is the risk for full siblings is greater than for half-siblings, and half-siblings have an increased risk over step and adopted siblings, which is comparable to the general population.\textsuperscript{8,9} Offspring from parents who both have MS are more likely to be affected than if only one parent has MS.\textsuperscript{3,8,10} A Dutch study reported that there is a significant maternal effect, where maternal half-siblings’ risk is not different from full siblings.\textsuperscript{3,11} From these population studies it is clear that a genetic component is involved in the development of MS, and researchers have been attempting to identify genetic markers that have the potential to predict disease susceptibility.\textsuperscript{12–14} The first to be recognized was the major histocompatibility complex (MHC), human leukocyte antigen (HLA) locus on chromosome 6p21.\textsuperscript{10,15,16} Genome wide association studies have confirmed the HLA DRB*1501 as a risk within the MHC locus as well as identifying roughly 14 other possible regions of risk including IL2RA and IL7RA. IL2R & IL7R are associated with regulatory T lymphocytes, which are strongly implicated in the immunopathogenesis of MS.\textsuperscript{15} Since 2011, the International Multiple Sclerosis Genetic Consortium has further advanced the number of known associated variants in MS to 110.\textsuperscript{13} The advancements in genetic studies are promising; however, they need to be considered alongside possible environmental factors, since genetics alone is unable to identify risk of disease definitively.\textsuperscript{12–14}

One of the current proposed environmental risk factors is vitamin D deficiency, due to the correlation between MS risk and latitude, implicating an inverse relationship
between sun exposure and risk of developing MS. The benefits of vitamin D have been demonstrated in the animal model of MS, experimental autoimmune encephalomyelitis (EAE). Active vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)\textsubscript{2} D), administered to mice with EAE resulted in disease amelioration. Moreover, vitamin D deficient mice experienced an accelerated onset of EAE compared to controls. This acceleration in disease was reversed when vitamin D supplements were administered. Additional studies have demonstrated that vitamin D is immunosuppressive, increasing regulatory T lymphocytes (Tregs) and suppressing pro-inflammatory cytokines (IL-17 and IFN\textgamma). Given this evidence, deficiencies in vitamin D may lead to increased risk of developing MS, and/or increased frequency of relapses or severity of MS.

One clear association with MS risk is exposure to the Epstein-Barr virus (EBV, human herpesvirus 4). Greater than 99% of individuals with MS are infected with EBV and MS is very rare in adults who are not infected with EBV. Individuals with higher titers of anti-EBV antibodies have an increased MS risk compared to individuals with low titers. While there is a consensus that EBV is linked to increased MS risk, there is insufficient evidence to show causality.

Currently, no one factor has been solely identified to cause MS. It is more likely that some combination of these factors leads to the development of MS. More research needs to be carried out to pursue the etiology of this disease further.
1.1.2 Subtypes of MS

In 1996, the National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis defined several different types of clinical disease. The different subsets were relapse-remitting (RR-MS), secondary progressive (SP-MS), primary progressive (PP-MS), and progressive relapsing (PR-MS). RR-MS is characterized by periods of neurological impairments followed by periods of remissions. Eighty-five percent of patients will experience RR-MS at disease onset, the majority of these patients will eventually develop SP-MS due to continued inflammatory attacks. PP-MS patients experience progressive deterioration from disease onset; with 10-15% of MS patients afflicted. In 2013, a committee formed by the NMSS, the European Committee for Treatment and Research in MS, and the MS Phenotype Group redefined the clinical courses of MS changing terminology from subtypes to phenotypes. For relapsing disease, clinically isolated syndrome (CIS) has been included with RR-MS, each of these phenotypes can be further distinguished by an active or non-active disease. CIS is defined as the first clinical onset of a potential MS diagnosis, affecting the optic nerves, brainstem or spinal cord. CIS transitions to RR-MS when the patient experiences subsequent clinical active events and meets current diagnostic criteria. Approximately two-thirds of CIS patients will develop RR-MS. The PR-MS subtype is no longer recognized as an independent form because of overlaps with PP and SP-MS. Therefore PP-MS (patients who experience progressive disability from onset of disease) and SP-MS (patients, who initially experienced the relapsing course transition to progressive disability) are the only phenotypes associated with progressive
disease. However, progressive disease is further characterized by the level of activity and progression. Although the updated clinical definitions are improvements in describing the course of MS, there is still much to be learned from clinical diagnostic testing that can enhance proper assessment of patients’ disease course.

1.1.3 Diagnostics

The McDonald MS Diagnostic Criteria provide clinicians with well-researched guidelines to assist them in diagnosing possible MS patients. It has been noted that these criteria should only be applied after other possible diagnoses have been ruled out and when the patient presents with clinically isolated syndrome (CIS). The diagnostic process involves clinical (attacks/presence of symptoms suggestive of MS) and paraclinical laboratory assessments, emphasizing demonstration of dissemination of lesions in space and time. Clinical assessment includes patient’s history, type of symptoms and the length of symptoms. Paraclinical assessments include MRI of the CNS to examine possible lesions, as well as examination of CSF looking for oligoclonal IgG bands or elevated IgG index.

1.1.4 Immunopathology

Multiple sclerosis is an autoimmune disease with inflammation occurring in the CNS and results in demyelination and eventual plaque formation. The hallmark pathological features of MS are demyelinating focal lesions found within the white and gray matter of the CNS, typically surrounding vasculature. Gross examination of MS brain tissue reveals well-defined plaques in white matter; in particular, optic nerves, periventricular regions, the brain stem and spinal cord. The immunopathology of
MS is not completely understood, though much has been learned from the examination of post-mortem MS tissue and animal models. Research has demonstrated that an inflammatory event of some magnitude precedes demyelination. This event consists of infiltration of the blood brain barrier (BBB) mainly by T lymphocytes and to a lesser extent B cells as well as macrophages.\textsuperscript{30,31} Once infiltration of the BBB has occurred resident glial cells become actively involved in the disease process that leads to the demyelinating lesions.\textsuperscript{30,32,33} It is accepted that MS begins with a T-cell mediated inflammatory process which leads to the formation of lesions and thus neurodegeneration.\textsuperscript{30} Our understanding of MS is complicated by the lack of homogeneity among MS patients. Nevertheless, researchers have been able to classify various pathological features of the disease including types of lesions and patterns of demyelination associated with different phases of MS.\textsuperscript{28–30,32,33}

White matter lesions are divided into four different types: classical active lesions, slowly expanding lesions, inactive lesions, and remyelinated shadow plaques.\textsuperscript{30} The classical active lesions are defined by lymphocytic inflammation and macrophage infiltration containing myelin debris.\textsuperscript{30,32} Slow expanding lesions are characterized by an inactive core surrounded by a thin rim of activated microglia and infiltrating macrophages, with little to no early myelin degradation products and some degree of axonal injury.\textsuperscript{30,32} Inactive plaques are distinct areas of demyelination devoid of activity and activated microglia, and these become more prevalent in late stages of the disease.\textsuperscript{30,32} Remyelinated, shadow plaques may be present at any stage of disease. However, they increase in number in very late stages of the disease.\textsuperscript{30}
The pathological features of cortical gray matter lesions vary depending on the stage of disease, relapsing or progressive. Three types of cortical gray matter lesions have been identified: cortico-subcortical compound lesions (affects gray and white matter), small intra-cortical lesions, and subpial lesions (displaying a band of superficial demyelination, that extends into deeper cortical layers). Progressive MS can be partially characterized by the more abundant subpial lesions, which are related to meningeal inflammation. The demyelination in subpial lesions is specific to MS pathology, and is not present in other CNS inflammatory diseases.

MS pathology is not limited to the sites of lesion formation; it also affects the normal appearing white matter (NAWM). Inflammation, microglia activation, astrocytic scaring as well as axonal injury and loss can be found within the NAWM. Late stages of disease progression result in notable tissue atrophy, enlarged cerebral ventricles and outer meningeal cerebrospinal fluid spaces.

Recent studies have indicated oxidative damage as a central mechanism contributing to tissue injury at all stages of disease and lesions, mediated by activated macrophages and microglia. Oxidative damage leads to mitochondrial injury which is present in active MS lesions. Injured mitochondria can explain many of the pathological features of MS including demyelination, oligodendrocyte apoptosis, axon injury, and loss as well as structural and functional disturbances of astrocytes and oligodendrocyte progenitor cells. It is thought that as the disease transitions to progressive MS, this oxidative damage gives rise to mitochondrial DNA damage which has been demonstrated to accumulate in neurons of patients with progressive MS.
The amount of knowledge that has been uncovered in the search for a complete understanding of the underlying cause and mechanisms of MS is vast; however, the complete pathophysiology involved remains elusive.

1.1.4.1 Lymphocytes

CD4⁺ T helper cells (Th1, Th2, Th17, Tregs) and CD8⁺ cytotoxic T cells are known to play a role in the pathogenesis of MS. Both cell types have been identified in MS lesions. In post-mortem examination CD8⁺ T cells outnumber CD4⁺ T cells, suggesting that cytotoxic T cells may play a larger role in axonal injury and lesion formation than CD4⁺ T cells. Animal models have been used for over 80 years to study the disease course of MS, and have given great insight into the possible immune mechanisms involved in disease development and progression. CD4⁺ T helper subsets have been distinguished from each other based primarily on the cytokine signature. Th1 cells produce IFNγ, Th2 cells produce IL-4, and Th17 cells produce IL-17. CD4⁺ Th1 cells were first identified as the main pro-inflammatory immune cell as a driving force for disease exacerbation in EAE. Th1 cells activate macrophages, in an healthy individual this would be helpful and not harmful. However, in the injured CNS of MS patients that have auto-reactive myelin Th1 cells, activation of macrophages against the myelin protein results in an attack of oligodendrocytes leading to demyelination.

In 1993, a new cytokine, IL-17, was identified. Since that time, extensive research has been done to examine its role in EAE and MS. The result has been the identification of another CD4⁺ Th cell lineage, Th17 cells. Th17 T cells secrete the
pro-inflammatory cytokines IL-17 and IL-6, and are regulated by IL-23. Studies examining human CSF found a greater percentage of IL-17 secreting cells compared to IFNγ in patients with MS compared to those without neurological deficits. IL-23 is important in the differentiation of Th17 cells and development of EAE, and IL-23 receptor signaling is required for the accumulation of Th17 cells in the CNS.

GM-CSF (granulocyte macrophage-colony stimulating factor) is another cytokine that was identified to be driven by IL-23 and secreted by Th17 cells. GM-CSF (CSF2) activates granulocytes, macrophages, epithelial cells, microglia, and monocytes and is essential in activating APCs during immune responses. In 2001, McQualter et al. utilized GM-CSF knock-out mice to determine the role of the cytokine in the development of EAE, and demonstrated the requirement of GM-CSF for the development of disease. Sheng et al., have hypothesized that GM-CSF+ Th cells represent a unique Th cell lineage. Further research is needed to fully understand the implications GM-CSF+ Th cells in the immunopathogenesis of MS.

Regulatory T cells (Tregs) have gained attention recently, in regards to their role in MS. Tregs are FOXP3+ (forkhead box P3) CD4+CD25+ T cells maintained by TGF-β. Tregs express IL-10 and TGF-β, which suppresses Th1 and Th17 cells. IL-10 is an anti-inflammatory cytokine that suppresses the expression of inflammatory cytokines, adhesion molecules and proteins essential for antigen presentation. When IL-10 was administered to mice with EAE, disease was prevented and/or attenuated. However, IL-10 also promotes tumor growth by inhibiting immune-mediated tumor regulation.
B lymphocytes within the spleen and lymph nodes, once activated, develop into immunoglobulin-secreting plasma cells, participate in antigen presentation to T lymphocytes, and secrete inflammatory cytokines that activate T lymphocytes and macrophages. These cells have also been implicated in disease progression and manifestation in MS, although to a lesser extent than T lymphocytes. One of the most prominent diagnostic features for the involvement of B cells is the presence of IgG oligoclonal bands in the CSF of MS patients. Approximately 90 percent of patients at the time of their first clinical event present with oligoclonal bands. Thus, CIS patients with positive IgG oligoclonal bands have a higher risk of developing MS.

1.1.4.2 Microglia and Macrophages

The innate immune cells of the CNS, microglia are tissue macrophages and are morphologically and phenotypically different from circulating macrophages. The function of microglia is to survey and protect the CNS environment. During times of CNS injury, microglia become activated and undergo morphological changes. Activated microglia are associated with and have a pivotal role in neuroinflammatory and neurodegenerative diseases, including MS. Macrophages are known to infiltrate the CNS along with other inflammatory cells and have been identified as playing an essential role in the disease process. Infiltrating macrophages and activated microglia are nearly indistinguishable from one another by histological analysis. Excessive activation of microglia results in the release of toxic substances such as nitric oxide, glutamate, free radicals and proteases, which can aid in neurodegeneration by contributing to oxidative damage and thus mitochondrial injury or damage.
1.1.4.3 Axonal Injury

All demyelinated lesions in MS display some amount of axonal injury.\textsuperscript{31,49,50} As lesions increase in size and number, and disease transitions from the acute to the chronic phase, axonal loss becomes apparent, resulting in irreversible neurological deficits.\textsuperscript{31,49–51} In fact, in chronic established lesions there is on average a 60-70 percent reduction in axonal density.\textsuperscript{49,52} Early in the disease process axons can be partially remyelinated; on an MRI areas of partially remyelinated plaques are known as shadow plaques.\textsuperscript{31,33,49,53} The remyelinated axons are recognized by the thin myelin sheath and increased internodal regions.\textsuperscript{30} The ability and extent of remyelination is variable between patients and CNS regions.\textsuperscript{30}

The cascade of events leading to axonal injury and neurodegeneration begins with chronic CNS inflammation.\textsuperscript{31} The release of toxic substances, such as reactive oxygen and nitrogen species (nitric oxide), cytokines, and glutamate creates a hypoxic environment.\textsuperscript{31} This hypoxia sustains oxidative stress leading to mitochondrial damage and dysfunction, demyelination, and increased calcium influx.\textsuperscript{31} It is important to note that axonal injury and degeneration is not limited to the demyelinated lesion but can also be seen to a lesser degree in the NAWM.\textsuperscript{31,49–51}

1.1.5 Disease Modifying Therapies

Currently, there are 10 FDA approved disease modifying therapies (DMT) available to MS patients, and 9 of the therapies are indicated for RR-MS.\textsuperscript{54} Mitoxantrone (Novantrone\textsuperscript{®}) is the only DMT indicated for use in declining RR-MS, PP-MS, and SP-MS. Other DMTs are natalizumab (Tysabri\textsuperscript{®}), teriflunomide (Aubagio\textsuperscript{®}),
fingolimod (Gilenya®), dimethyl fumarate (Tecfidera®), interferon beta 1b (Betaseron® and Extavia®), interferon beta 1a (Avonex® and Rebif®), and glatiramer acetate (Copaxone®). Three of the approved therapies are oral while the others are either self-injected or given intravenously.

Glatiramer acetate promotes differentiation of Th2 and Treg cells, leading to bystander suppression in the CNS, increased release of neurotrophic factors from immune cells and deletion of myelin-reactive T cells. Side effects associated with glatiramer acetate include injection site reactions, chest pain, vasodilation, rash and dyspnea and potential effects of immune responses.

Interferon beta is available in two forms: interferon beta 1a and interferon beta 1b. Both drugs promote the shift from Th1 to Th2 cells, reduce BBB trafficking, restore Tregs, inhibit antigen presentation and enhance apoptosis of autoreactive T-cells. However, side effects of interferons include flu-like symptoms, injection site reactions, elevated liver enzymes and decreased white blood cell counts (WBC). Other severe side effects that reduce patient compliance include risk of developing depression, suicide and psychosis, liver damage, allergic reactions, necrosis at the site of injection, decreases in peripheral blood cell counts, likelihood of seizures and possible development of other autoimmune diseases.

Dimethyl fumarate, the most recently FDA approved oral therapy, promotes anti-inflammatory and cytoprotective activities mediated by the NRF2 (NFE2L2) pathway. Dimethyl fumarate side effects include flushing, gastrointestinal related symptoms, pruritis, rash and erythema and in rare cases lymphopenia.
Fingolimod is an oral therapy that is proposed to block sphingosine-1-phosphate receptors (S1PR1) on lymphocytes preventing migration from secondary lymph organs.\textsuperscript{57,58} Side effects include headache, influenza, diarrhea, back pain, elevated liver enzymes, bradycardia during the first dose, macular edema, lymphopenia, and in rare cases bronchitis or pneumonia.\textsuperscript{54}

Teriflunomide, an orally available therapy, is proposed to induce a cytostatic effect on rapidly dividing peripheral lymphocytes, as well as inhibiting \textit{de novo} pyrimidine synthesis.\textsuperscript{54,59} Side effects are alopecia, diarrhea, influenza, nausea, paresthesia.\textsuperscript{54,59} Severe effects and warnings include hepatotoxicity, risk of teratogenicity, decreased neutrophils, lymphocytes and platelets, risk of tuberculosis, potential increased risk of malignancy, peripheral neuropathy, acute renal failure, treatment-emergent hyperkalemia, increased renal uric acid clearance, interstitial lung disease, and Stevens-Johnson syndrome, as well as elevated blood pressure.\textsuperscript{54,59}

Mitoxantrone, indicated for use only in deteriorating RR and SP-MS, disrupts DNA synthesis and repair, inhibits proliferation of B and T cells as well as macrophages, impairs antigen presentation and secretion of IFN\textgreek{y}, TNF\textgreek{a}, and IL-2.\textsuperscript{54,55,60} Side effects include a temporary blue discoloration of sclera and urine, nausea, alopecia, menstrual disorders, possible infertility, increased infections, arrhythmias, abnormal EKG and congestive heart failure.\textsuperscript{54,55,60} Warnings include cases of severe local tissue damage (extravasation), cardiotoxicity, acute myelogenous leukemia and myelosuppresion.\textsuperscript{54,55,60}
Natalizumab blocks α4-integrin on lymphocytes which reduces trafficking into the CNS.\textsuperscript{54,60} Mild side effects include headache, fatigue, urinary tract infection, lower respiratory tract infection, arthralgia, urticaria, gastroenteritis, vaginitis, depression, and diarrhea.\textsuperscript{54,60,61} Severe side effects include PML, liver damage, herpes encephalitis and meningitis as well as hypersensitivities.\textsuperscript{54,60,61}

All available therapies have undesirable side effects; some have the potential of developing severe effects such as progressive multifocal leukoencephalopathy (PML).\textsuperscript{54} No therapy cures the disease or is 100\% effective in reversing disease progression. The cost burden associated with MS is devastating to the patients and their families, and direct costs alone from drug treatments alone can approach $40,000 annually.\textsuperscript{2,61,62} Therefore it is not surprising that patient compliance for the existing DMTs ranges between 40\% to 80\%.\textsuperscript{63}

1.2 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the most widely used animal model to study MS, due to its similarities in immunopathology associated with MS. The origin of EAE dates back 80 years, when Koritschoner and Schweinburg\textsuperscript{39,64} used human spinal cord to inoculate rabbits producing inflammation in the spinal cord.\textsuperscript{39,64} Several years later, Rivers and associates made attempts to reproduce complications from Louis Pasteur’s rabies vaccines by subjecting monkeys to repeated (up to 80 injections over a year) immunization with emulsions of rabbit brain.\textsuperscript{39,64,63} The experiments resulted in histological findings in the brain and spinal cord consisting of perivascular infiltration and myelin degeneration.\textsuperscript{39,64,65} With the development of
complete Freund’s adjuvant (CFA) in 1942, EAE was induced after a single injection. Utilizing this new immunization method, EAE was induced in a wide range of species. EAE studies revealed pathological features resembling human autoimmune demyelinating diseases, in particular, MS. EAE has been utilized in the development of MS therapies, including glatiramer acetate, mitoxantrone, and natalizumab. Although EAE is the best characterized model of MS, it still has limitations and many therapies that had positive results in the model failed to have clinical efficacy.

There are now well characterized models available to study specific aspects of the immunopathogenesis of MS, ranging from actively induced animal models to adoptive transfer and transgenic spontaneous models. Actively induced disease models utilize myelin proteins that have been identified in MS, including myelin oligodendrocyte glycoprotein (MOG$^{35-55}$), proteolipid protein (PLP$^{139-151}$) and myelin basic protein (MBP). Different forms of the disease can be produced depending on animal strain, choice and the amount of antigen utilized. The most common active induction models include the C57BL/6 mouse immunized with MOG$^{35-55}$ emulsified in CFA followed by injections of pertussis toxin producing a chronic monophasic disease, and the SJL/J mouse immunized with PLP$_{139-151}$ emulsified in CFA followed by injections of pertussis toxin inducing relapse-remitting EAE.

The adoptive transfer animal model induces EAE through peripheral introduction of autoreactive CD$^+$ T cells to a naïve mouse. The most recent advances in EAE are
the development of transgenic mice that over express myelin-specific T cell receptors (TCR) on CD4$^+$ T cells and/or CD8$^+$ T cells, these mice develop spontaneous EAE.\textsuperscript{70,72}

1.3 Opioids

Opioids have been utilized for both their analgesic and euphoric properties, dating back as early as 4,000 BC in the Middle East.\textsuperscript{73} The first opiate was opium, derived from the opium poppy.\textsuperscript{73} In 1804, the active ingredient in opium was isolated and named morphine.\textsuperscript{73} Seventy years later heroin was invented and used as a cough suppressant until the addictive properties were realized and by 1924, opioids were banned from nonmedical products.\textsuperscript{73} By 1973, opioid receptors were discovered, and just two years later Hughes and colleagues discovered two endogenous opioids, leu- and met$^5$-enkephalin.\textsuperscript{74–78}

1.3.1 Endogenous Opioids

Since the discovery by Hughes, other endogenous opioid peptides have been characterized, including endorphins and dynorphins. Each peptide is derived from specific precursors, prepro-opiomelanocortin (POMC, giving rise to β-endorphin), preproenkephalin (giving rise to the enkephalins) and preprodynorphin (giving rise to dynorphins).\textsuperscript{79} Endorphins are polypeptides, and have a high affinity for μ-opioid receptors and to a lesser extend δ- and κ-opioid receptors.\textsuperscript{79} β-endorphins, located predominantly in the hypothalamus and pituitary, are involved in alleviating pain, as well as reducing anxiety and can stimulate euphoria.\textsuperscript{79} Dynorphins, found in various areas of the brain, brainstem, and spinal cord, exert their effects primarily through the κ-opioid receptor.\textsuperscript{79} Enkephalins are pentapeptides present throughout the body, including the
Met- and leu-enkephalin both have high affinity to bind to δ-opioid receptor and a lesser extent μ-opioid receptors. Met- and leu-enkephalin both have high affinity to bind to δ-opioid receptor and a lesser extent μ-opioid receptors. 

1.3.2 Opioid Receptors

In 1973, opioid receptors were discovered in response to the pharmacological evidence of opioid antagonists. Pert and Snyder radiolabeled naloxone, a non-selective opioid antagonist, and identified opioid receptors and their location in mammalian brain. Since then, opioid receptors have been extensively studied, recognized to exist in two classes of “classical” and “non-classical” opioid receptors. The three identified classical opioid receptors, μ- (MOR, OPRM1), δ- (DOR, OPRD1), and κ- (KOR, OPRK1), have been well characterized. Classical opioid receptors are G-protein coupled 7-transmembrane receptors that activate inhibitory G protein pathways. They are involved in analgesia, reward, depression, anxiety and addiction. The “non-classical” opioid receptor family includes nociceptin receptor (ORPL1, based solely on genetic similarities) and the opioid growth factor receptor (OGFr). OGFr is pharmacologically similar to classical opioid receptors, in that it’s inhibitory growth regulation can be blocked by opioid antagonist (naloxone and naltrexone), but differs in structure, amino acid sequence, and location.

1.3.3 Opioid Antagonists

Two opioid antagonists, naloxone and naltrexone block the effects of opioid receptor-peptide interactions by binding to the receptor and interfering with agonist binding. Both antagonists are non-selective for the classical opioid receptors as well as the OGFr. Naloxone and naltrexone are structurally and functionally similar.
However, naltrexone has a greater oral bioavailability and a longer half-life. Making it more suitable for therapeutic interventions. In 1984, the FDA approved the naltrexone HCl for the treatment of opioid addiction. More recently, off label uses for low doses of naltrexone (LDN, daily dosages 1/10th of typical dose prescribed for opioid addiction) are being utilized in the treatment regimen for autoimmune diseases such as MS. LDN is involved in analgesia, anti-inflammatory actions and upregulates endogenous opioids. LDN transiently blocks opioid receptors which causes the body to upregulate endogenous opioids and opioid receptors. LDN has been utilized in clinical trials for treatment of Crohn’s disease, as well as MS. Crohn’s disease has received the most attention in reference to the ability of LDN to alleviate symptoms. While, clinical trials associated with MS have been limited in number and size of study. More research is required to determine whether LDN is having an effect on new lesion formation or inhibiting growing lesions.

1.3.4 OGF-OGFr axis

In the 1980’s it was postulated that endogenous opioid peptides function in growth regulation of normal and abnormal cells and tissues. Almost a decade later, met⁵-enkephalin was identified to be the most potent opioid peptide associated with growth. Met⁵-enkephalin has since been referred to as opioid growth factor (OGF) in order to distinguish its role on growth regulation. It was determined that this effect was not due to interactions with the classical opioid receptors. However, antagonists studies using naloxone demonstrated that naloxone can inhibit the negative growth effects of OGF, which suggests that OGF is interacting through an opioid receptor.
order to find the specific receptor through which OGF exerts its growth regulation, binding studies were utilized with radio-labeled OGF. After extensive research, a single receptor was identified that mediates OGF activity, the opioid growth factor receptor (OGFr). Since its discovery, OGFr has been isolated, characterized, cloned and sequenced in mouse, rat, and human tissue. In addition, OGFr gene expression has been identified in most fetal and human tissues. To date, it is presumed that OGF and OGFr are present in all tissues, as all that have been evaluated displayed some level of expression. Further research into the OGF-OGFr axis has led to the proposed mechanism, which begins with active clathrin-mediated endocytosis of OGF. Once inside the cell OGF binds to OGFr on the outer nuclear membrane, and the complex associates with karyopherin β and Ran to translocate through a nuclear pore into the nucleus. This results in upregulation of p16\(^{\text{INK4a}}\) and p21\(^{\text{WAF1/CIP1}}\) pathways which mediate a decrease in proliferation, by delaying the cell cycle in the G1/S phase. In normal and cancer tissues the OGF-OGFr axis, when activated, has been shown to be a potent inhibitor of cellular proliferation. This tonically active inhibitory axis can be blocked by the opioid antagonist naltrexone (NTX) resulting in accelerated cell proliferation. In the early 1980’s it was determined that the length of receptor blockade was related to the response in cell proliferation, such that long-term blockade enhanced proliferation while short-term blockade had an inhibitory effect on proliferation. Manipulation of the OGF-OGFr axis has positive implications for
diseases such as MS and other autoimmune diseases, cancers, and diabetic wound closure.\textsuperscript{76,84,92,93}

Additional studies have demonstrated the beneficial effects of modulating the OGF-OGFr axis in chronic EAE through administration of either OGF or low doses of naltrexone (LDN).\textsuperscript{92,94–97} Administration of OGF both at the time of immunization and disease induction was shown to decrease or completely abolish clinical signs of disease as well as reduce the histopathological signs of disease.\textsuperscript{92,94–97} Intermittent blockade of the OGF-OGFr axis in the chronic EAE model beginning at disease induction resulted in similar results.\textsuperscript{92,94} Histopathological findings in these studies displayed decreased demyelination, infiltrating T cells, activated astrocytes, activated microglia/macrophages as well as decreased cellular proliferation, specifically reductions in astrocytic proliferation.\textsuperscript{92,94–97} Campell et al., further demonstrated that the decrease in astrocyte proliferation was due to the OGF-OGFr axis, through \textit{in vitro} studies.\textsuperscript{98}

\subsection*{1.3.5 Opioids and the Immune System}

It is recognized that classical (MOR, DOR, & KOR) and non-classical (OGFr) opioid receptors are present on immune cells, in humans and murine cell lines.\textsuperscript{99–106} Studies, have indicated, however, that of the classical opioid receptors, DOR and KOR are the dominant opioid receptors on T cells.\textsuperscript{104} KOR is expressed to a greater extent in lymph compartments which contain immature immune cells, while both DOR and KOR expression are enhanced in activated cells.\textsuperscript{104} The initial recognition of possible opioid receptors on immune cells stemmed from experiments performed by Wybran in 1979.\textsuperscript{104,107} Their research demonstrated that morphine and met\textsuperscript{5}-enkephalin have
opposing direct effects on immune function via rosetting in human T lymphocytes in response to sheep blood cells.\textsuperscript{104,107} Met\textsuperscript{5}-enkephalin has been shown to inhibit proliferation of anti-CD3 stimulated murine splenic CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes, as well as diminish IL-2 secretion when present in higher concentrations (10\textsuperscript{-8}-10\textsuperscript{-6} M) in the media.\textsuperscript{104} They attributed these effects to the classical opioid receptor delta, given similar results with the selective DOR agonist DADLE ([D-Ala\textsubscript{2}-D-Leu\textsubscript{5}]-enkephalin).\textsuperscript{104} Zagon and Donahue have demonstrated the ability of OGF, administered \textit{in vitro} to stimulated splenic T and B lymphocytes, to suppress cellular proliferation; OGF had no influence on naïve lymphocytes.\textsuperscript{105,106} Utilizing siRNA knockdowns, it was demonstrated that the effects noted by OGF were indeed mediated through the OGFr and not a classical opioid receptor.\textsuperscript{105,106} Naltrexone, a non-specific opioid antagonist, did not alter lymphocyte proliferation of stimulated T and B lymphocytes.\textsuperscript{105,106} Therefore demonstrating that the OGF-OGFr axis is indeed intact in T and B lymphocytes.\textsuperscript{105,106}

Further evidence of the role of OGF involvement in immunomodulation was demonstrated when researchers, examining human colorectal cancer, found that met\textsuperscript{5}-enkephalin suppresses T lymphocyte proliferation in a dose-dependent manner.\textsuperscript{108} They also noted that met\textsuperscript{5}-enkephalin was especially inhibitory on CD4\textsuperscript{+} T lymphocytes.\textsuperscript{108} This is supportive of further research into how OGF can be utilized to alter autoimmune disease progression.

The precursor of OGF, preproenkephalin, has been implicated to play a role in immunomodulation as well.\textsuperscript{109} Hook et al., 1999, found that preproenkephalin mRNA
was expressed at higher levels in Th2 compared to Th1 cells \textit{in vitro}.\textsuperscript{109} They also demonstrated that CD4\textsuperscript{+} T cells isolated from preproenkephalin null mice could differentiate into both Th1 and Th2 effector cells in culture, suggesting that preproenkephalin is not a requirement for CD4\textsuperscript{+} Th differentiation.\textsuperscript{109} In 1991, Kuis et al., with the knowledge that preproenkephalin mRNA expression is found in murine B and T cells, wanted to determine which human blood mononuclear cells expressed preproenkephalin mRNA, how it was regulated, and which peptides are produced.\textsuperscript{110} They found that human peripheral blood mononuclear cells do express preproenkephalin mRNA and that monocytes, not T cells, were capable of processing proenkephalin to met\textsuperscript{5}-enkephalin.\textsuperscript{110}

\textbf{1.3.5.1 Opioids and MS/EAE}

In 1987, Janković and Marić reported that EAE rats receiving either 5 mg/kg met\textsuperscript{5}-enkephalin or 0.2 mg/kg met\textsuperscript{5}-enkephalin experienced a disease course inversely related to the dosage of met\textsuperscript{5}-enkephalin received.\textsuperscript{111} The higher dose of met\textsuperscript{5}-enkephalin ameliorated the disease course while lower dose of met\textsuperscript{5}-enkephalin exacerbated disease.\textsuperscript{111} This is in agreement with studies published by our lab that treatment of EAE with 10 mg/kg OGF (met\textsuperscript{5}-enkephalin) improves clinical and morphological disease.\textsuperscript{92,94–97} In 2006, Weir and colleagues examined the role of the OGF precursor, preproenkephalin, in EAE using preproenkephalin deficient mice (\textit{Penk\textsubscript{rs\textsuperscript{tm1Pig}}, PPNK-d}).\textsuperscript{112} They found that PPNK-d mice experienced a delayed disease onset, displayed a decrease in disease incidence and decreased severity at acute disease compared to C57BL/6 wild-type mice.\textsuperscript{112} Interestingly, they found that
proliferation was not altered in T cells isolated from PPNK-d mice compared to the wild type controls.  However, PPNK-d mice displayed fewer antigen specific Th1 cells as well as a decrease in production of the proinflammatory cytokine, IFNγ.  In addition, histopathological examination of the brain and spinal cords of PPNK-d mice exhibited decreased infiltration of mononuclear cells and reduced demyelination at early time points.  This is interesting as it would suggest that endogenous OGF levels, when absent could diminish EAE exacerbation. However, in order to fully understand the implications of the findings reported by Weir et al., continued research is required to elucidate the mechanism and how this information will impact the therapeutic options for MS patients.

Peptidases, such as aminopeptidase N (CD13), degrade endogenous opioids, in particular OGF, in addition to playing a role in the activation of T cells.  Reinhold et al., 2011, took advantage of these properties by utilizing a peptidase inhibitor, PETIR™, that inhibits aminopeptidase N and dipeptidyl peptidase IV (DP IV), in the treatment of EAE in SJL/J mice.  They demonstrated that PETIR™ ameliorated disease, further supporting the important role of the OGF-OGFr axis in EAE and/or MS.

1.4 Gap in Knowledge

MS is one of the most common CNS debilitating diseases. The current therapies available are expensive, focus on narrow aspects of disease modification, and have undesirable side-effects. There exists a need for cost-effective therapy options for MS patients. Modulation of the OGF-OGFr axis, by administration of OGF or LDN, in both chronic and/or chronic established EAE diminishes disease severity and reduces the
histopathological damage associated with the disease. This holds promise for those patients who experience the progressive form of the disease, but 85 percent of MS patients experience the relapse-remitting disease course. It is unknown whether OGF and/or LDN will be an effective treatment option for RR-MS patients. Thus, there is an unmet medical need to understand the role of the OGF-OGFr axis in relapse-remitting EAE.

1.5 Hypothesis and Specific Aims

The central hypothesis of this dissertation is that modulation of the OGF-OGFr axis alters the course of relapse-remitting EAE. The objectives of this dissertation are to establish a working model of RR-EAE utilizing immunization of the SJL/J mouse with PLP\textsubscript{139-151} and to assess the safety and efficacy of LDN or OGF when treatment is initiated at the time of induction or at the time of established clinical disease. Behavior and morphology of glia, macrophages, and neurons will be evaluated, as well as the effects of treatment regiments on CD4\textsuperscript{+} T lymphocyte infiltration into the CNS.

The hypothesis will be tested by completing the following specific aims.

**Specific Aim 1:** Determine whether altering the OGF-OGFr axis through daily OGF administration alters the progression of RR-EAE. This aim will establish a working model of RR-EAE utilizing the SJL/J mouse and proteolipid protein 139-151 (PLP\textsubscript{139-151}) and treat mice with OGF beginning at the time of disease induction. Behavior and spinal cord histopathology will be assessed by examination of T lymphocytes, activated astrocytes, microglia, proliferation, demyelination and neuronal damage in saline-treated and OGF-treated RR-EAE mice.
Specific Aim 2: Ascertain whether modulation of the OGF-OGFr axis through daily administration of OGF alters clinical disease in established RR-EAE. This aim will involve examining OGF treatment initiated two days after clinical disease is observed, through daily behavioral observations and examination of lumbar spinal cord pathology at peak disease, time of first relapse and long-term disease. Spinal cords will be stained with markers for T lymphocyte infiltration, macrophage/microglia, activated and proliferating astrocytes, and demyelination.

Specific Aim 3: Determine whether intermittent blockade of the OGF-OGFr axis through daily administration of LDN alters clinical disease in mice with established RR-EAE. This aim will investigate the effects of intermittently blocking the OGF-OGFr axis on established disease through daily behavior observations and analysis of spinal cord pathology after 5, 14 and 40 days of treatment with LDN. Spinal cords will be stained with markers for T lymphocyte infiltration, macrophage/microglia, activated and proliferating astrocytes, and demyelination.

Specific Aim 4: Define whether modulation of the OGF-OGFr axis through daily administration of OGF or intermittent blockade with LDN alters the number of CD4+ T lymphocytes and associated effector cells within the CNS in established PLP-EAE. This aim will utilize intracellular cytokine staining of mononuclear cells from the CNS of EAE mice that have been treated with saline, OGF or LDN for 5 days. Mice with PLP-EAE will be evaluated to assess effects of modulating the OGF-OGFr axis on the distribution of effector CD4+ T lymphocytes present in the CNS at peak disease. The CD4+ Th cells to be examined are Th1 (IFNγ), Th2 (IL-4), Th17 (IL-17A), and Tregs (Foxp3).
Currently, some MS patients have requested LDN therapy for the alleviation of their symptoms because it is an oral compound that is safe and inexpensive; however, no large clinical study has been completed on OGF or LDN, alone or in combination with standard care, for progressive or RR-MS. Data from these studies will support our long-term goal to improve treatment options for MS patients.
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CHAPTER 2. OGF TREATMENT AT INDUCTION OF RELAPSE-REMITTING EAE
**Rationale**

Exogenous OGF attenuates the course of established chronic EAE in the C57Bl/6 mouse model that utilizes the MOG\textsubscript{35-55} peptide for immunization. Approximately 10% of multiple sclerosis patients present with primary progressive MS, which is comparable to chronic EAE, while 85% of patients present with relapse-remitting MS. The goal of this aim is to determine whether OGF treatment attenuates the behavioral course of RR-EAE when administered at time of immunization and determine the effects of OGF on the histopathology found within the lumbar spinal cord.
Treatment of a Relapse-Remitting Model of Multiple Sclerosis with Opioid Growth Factor

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Abstract

Relapse-remitting multiple sclerosis (MS) is an immune-mediated disease of the central nervous system that affects more than 2.5 million individuals worldwide. While the etiology of MS is unclear, disease manifestation involves proliferation and activation of lymphocytes and astrocytes, leading to demyelination and neuronal damage. Current therapies are not completely effective, and few target the underlying pathophysiology of MS. The purpose of this study was to examine the therapeutic efficacy of a novel biological pathway, the opioid growth factor (OGF) – OGF receptor (OGFr) axis. OGF inhibits DNA synthesis and has been shown to repress proliferation of T lymphocytes, microglia, and astrocytes in other autoimmune disorders. An animal model for relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) was established by immunization of SJL/J mice with proteolipid protein. Treatment with OGF or saline was initiated simultaneously with immunization, and within 9 days, behavioral signs of RR-EAE were observed. OGF-treated RR-EAE animals had less severe clinical disease than mice receiving saline and exhibited 66% reductions in median cumulative disease scores, as well as prolonged periods of remission and diminished number and length of disease relapses. Neuropathological examination of lumbar spinal cord revealed reductions in the number of T lymphocytes, microglia/macrophages, and activated astrocytes, with cell proliferation being the mechanism targeted by OGF. Areas of demyelination and neuronal damage were markedly reduced during the 55-day observation period. These data are the first to demonstrate that OGF prevented
relapses in RR-EAE and diminished underlying neuropathology, corroborating the potential of the OGF – OGF receptor pathway for treatment of MS.
Introduction

Multiple sclerosis (MS) is a chronic, debilitating immune-mediated disease of the central nervous system (CNS) that affects more than 2.5 million individuals worldwide, with nearly 85% of the patients afflicted with the relapse-remitting form (RR-MS).\(^1\) Disease manifestation involves proliferation and activation of T-lymphocytes, microglia, and astrocytes, leading to inflammation, demyelination, and axonal damage. Over a period of time, neurodegeneration in the spinal cord and brain are associated with disease progression. Current therapies are designed to target one or more of the symptoms of the disease, but few are disease-modifying in nature.\(^2\) Despite some differences in the cause of relapse-remitting disease, the mouse model of relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) represents an animal model that responds to proteolipid protein immunizations by proliferation of T-cells and microglia, and activation of astrocytes.\(^3,4\) The behavioral course of disease can be charted and utilized as an endpoint for therapeutic interventions.

Opioid growth factor (OGF), chemically termed [Met\(^5\)]-enkephalin, and its receptor, OGFr, form a physiological pathway that maintains homeostasis and can be modulated to shift the course of disease.\(^5,6\) Modulation of the OGF-OGFr axis either by chronic treatment with the endogenous peptide OGF, or by upregulation of OGF and OGFr following low dosages of naltrexone (LDN), in mice immunized with myelin oligodendrocytic glycoprotein (MOG) to establish progressive EAE was neuroprotective against encephalitogenic processes.\(^7,8,9\) Signs of behavioral deficits are delayed in appearance, reduced in severity, or reversed in EAE mice receiving 10 mg/kg OGF.
beginning at the time of disease induction in comparison to mice receiving daily injections of saline.\textsuperscript{8,9,10} Evaluation of lumbar spinal cord sections revealed significant reductions in the number of activated astrocytes and regions of demyelination.\textsuperscript{8,9} Treatment of mice with exogenous OGF initiated at the time of established EAE reversed the progression of clinical disease within 6 days.\textsuperscript{10} Mice with MOG-induced EAE and receiving OGF treatment initiated with established disease exhibited a reduced number of activated astrocytes and damaged neurons, decreased areas of demyelination, and repressed T cell proliferation.\textsuperscript{10} Within 3 weeks of MOG immunization, EAE mice treated with saline had 3.5-fold elevated numbers of iba1\textsuperscript{+} cells in the lumbar spinal cord in comparison to normal mice. OGF-treated EAE mice had 30\% reductions in the number of microglia/macrophages relative to EAE mice receiving saline.\textsuperscript{10} OGF therapy reduced the number of T lymphocytes in the spinal cord (detected by CD3 staining) by 56\% relative to EAE mice receiving saline. The mechanism targeted by OGF was cell proliferation, with Ki67 staining markedly reduced in spinal cord sections from OGF-treated EAE mice. Sections stained with both Ki67 and GFAP revealed only 3\% of cells in OGF-treated EAE mice being double labeled in comparison to ~14\% of cells in spinal cord sections from saline-injected EAE mice. OGF has been shown in a number of models to up-regulate cyclin-dependent inhibitory kinases and protract cell passage from $G_0G_1$ to S.\textsuperscript{11} This mechanism to reduce cell proliferation has been documented for T and B lymphocytes stimulated in vitro to replicate.\textsuperscript{12,13}

These observations were demonstrated using an animal model of MOG-induced EAE that most resembles chronic, progressive MS; however, most patients have RR-
In the present study, we established a mouse model of RR-EAE using proteolipid protein 139-151 (PLP_{139-151}) immunization of SJL/J mice, and determined the efficacy of daily injections of OGF initiated at the time of disease induction. Mice were observed daily over a 55 day period of time, and lumbar spinal cord tissue was collected on 10, 14, and 55 days after initiation of treatment in order to assess expression and proliferation of astrocytes, T lymphocytes, microglia/macrophages, as well as demyelination and neuronal damage.
Materials and Methods

Mice

Female SJL/JOrlCRL mice (Charles River Labs, Wilmington, MA) were housed 5 per cage under standard conditions in a separate room from other rodents and acclimated for one week prior to disease induction; food and water were available *ad libitum*. As the course of EAE disease progressed, soft food and water packets were placed on the floor of the cages. All experiments were conducted in accordance with the National Institute of Health guidelines on animal care, and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Induction of Relapse-Remitting EAE and Treatment

RR-EAE was induced by immunization with proteolipid-protein (PLP<sub>139-151</sub>) following established protocols. Mice were given multiple injections subcutaneously (total volume 300 μl) on the back with an emulsion of 100 μg PLP<sub>139-151</sub> (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); equal volumes of the PBS containing PLP<sub>139-151</sub> and Freund’s adjuvant were emulsified by vortexing. Intraperitoneal (i.p.) injections of 200 ng pertussis toxin in phosphate buffered saline (List Biological Laboratories, Campbell, CA) were given on days 0 and 2. Mice were lightly anesthetized with 3% isoflurane (Vedco, Inc., St. Joseph, MO) for PLP<sub>139-151</sub> and *M. tuberculosis* injections; anesthesia was not
required for daily OGF or saline treatments. Normal mice received equal volumes of sterile phosphate buffered saline in place of PLP$_{139-151}$ and pertussis toxin.

Mice immunized with PLP$_{139-151}$ were randomized to receive daily intraperitoneal injections (0.1 ml) of either 10 mg/kg OGF (Polypeptide Laboratories, Torrance, CA) (PLP+OGF) or an equal volume of sterile phosphate-buffered saline (PLP+Saline) administered to mice beginning on the day of immunization. All injections were given between 9.00 and 10.00 hr. Animals were weighed weekly in order to adjust drug dosages.

**Behavioral Observations**

All mice were observed by 2 individuals, with one evaluator masked to the treatment group. Behavior was scored by placing each animal on a smooth surface and recording tail tonicity, gait and righting reflex. Limb strength was assessed by inverting the animal on a wire grid and observing the ability for each mouse to maintain grasp. To accommodate a broad series of behavioral observations, a modified scale of 0 to 10 (10 = death) was utilized, with scoring based on summation of gradations for intermediate behavior observed for tail tonicity, gait, righting reflex, and individual limb tonicity. Paralysis of a limb was recorded when the limb was unable to support body weight.

Disease onset was considered the second consecutive day that a mouse had a behavioral score of 0.5 or greater. Cumulative disease scores for each treatment group were the summation of behavioral scores for all mice throughout the 55-day experimental period. Disease index was calculated as the cumulative score divided by the day of disease onset. Mean maximal severity scores were calculated as the
average of the highest disease score for each mouse over the course of the experiment. A complete remission was considered when the animal returned to a score of 0.5 or less for two consecutive days. A relapse was defined as the period of time when behavioral scores for an individual mouse over 2 days were greater than or equal to two points higher than their average running behavioral score. Behavioral scores for mice that survived the entire 55 days were included in analyses; behavioral scores for mice that were euthanized for pathology were recorded, but not included in the long-term behavioral assessment.

**Neuropathology**

Spinal cord tissues were collected from mice in all groups at 10, 14, and 55 days of treatment which represented time prior to clinical signs of disease, the time of peak disease, and approximately 8 weeks of drug treatment, respectively. Prior to being euthanized by intraventricular perfusion, mice were deeply anesthetized with a cocktail containing ketamine (30 mg/kg), xylazine (5 mg/kg) and acepromazine (2 mg/kg) diluted in sterile phosphate buffered saline. After perfusion with fresh 4% paraformaldehyde, intact vertebral columns were dissected and post fixed in 4% paraformaldehyde for 18 hours. Frozen sections (10 µm) of the lumbar region (L4-L5) were stained for microglia/macrophages\textsuperscript{17}, T lymphocytes,\textsuperscript{18,19} and activated astrocytes.\textsuperscript{20} Paraffin sections (10 µm) of the lumbar region (L5-L6) were stained as described below for demyelination\textsuperscript{21} and markers of neuronal damage.\textsuperscript{14,22} Cell numbers, as well as areas of demyelination, were analyzed using published methodology.\textsuperscript{7-10}
Overall cell proliferation, as well as specific astrocyte proliferation, was evaluated by Ki67 staining. Controls for immunostaining included sections stained with secondary antibody only. At least 2 sections/mouse from 3-8 animals/group were evaluated for each histopathological measure.

Microglia/macrophages were identified using iba-1 antibody (1:200, Wako, Osaka, Japan); the number of positively stained cells was counted in 2-4 fields (0.26 mm²) on the right and left sides of the central canal. A positive microglia/macrophage was considered to be a nucleus surrounded by cytoplasm and having at least one projection; iba-1 immunoreactivity was evident surrounding the nucleus.

T lymphocytes were identified using CD3 antibody (1:200, ab5690, Abcam, Cambridge, MA). Cells were counted in multiple fields (0.26 mm²) of central ventral white matter.

Astrocytes were identified using glial fibrillary acidic protein (GFAP) antibody (1:500, Dako, Carpinteria, CA), and their number recorded in regions surrounding the central canal.

**Demyelination and Neuronal Damage**

Demyelination was evaluated using Image-Pro Plus 6.2 software (MediaCybernetics, Bethesda, MD) by inspection of tissues stained with Luxol fast blue (Roboz Surgical Instrument Co., Washington, DC)-neutral red (Fisher Scientific, Pittsburgh, PA). Neuronal damage was quantitated in the ventral horn of the lumbar spinal cord by recording the number of neurons in cresyl violet stained sections.
Mechanistic Pathways Targeted by OGF

Proliferation was assessed by staining with anti-Ki67 (1:200, ab66155, Abcam). Ki67 protein is restricted to cells undergoing active phases of the cell cycle, and is absent from G₀ resting cells. To evaluate proliferating astrocytes, serial sections were stained with GFAP and Ki67 and images from adjacent sections of central ventral white matter were digitally overlaid for analysis.

Statistical Analyses

Behavioral scores were analyzed using the Mann-Whitney non-parametric test, and values were expressed as median with interquartile ranges (IQR). Parametric behavioral data, as well as morphological data, were analyzed using the 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

General Observations and Behavior

Two separate experiments were conducted and all mice inoculated with PLP developed signs of EAE in both studies. Injections of PLP\textsubscript{139-151} resulted in redness and swelling at the site of injection in some SJL mice; however, no mouse died from immunization procedures (i.e., first 11 days). In one study, 3 of 30 mice died at or near the peak disease (i.e., days 12-14), with no additional deaths recorded. No normal mouse developed any neurological abnormality or died over the course of 2 months. Fifteen saline-treated and 18 OGF-treated mice were included in the behavioral studies.

PLP\textsubscript{139-151} immunizations resulted in the first appearance of clinical signs of RR-EAE on day 9, with the average day of disease onset being day 11; onset was comparable between OGF- and saline-injected mice as shown in Figure 2.1A, a representative graph of the initial study on RR-EAE behavior. Within 4 days of disease onset (i.e., day 13), saline-treated mice displayed their highest clinical score correlating to limp tail, wobbly gait, and partial limb paralysis, whereas the clinical behavioral scores for OGF-injected mice reached a median score of only 3 on days 14-16 and 19. Median (± IQR) behavioral scores for OGF-treated mice with RR-EAE were significantly lower than those of mice receiving saline on sixteen days during the observation period, with median values for PLP+OGF mice reaching zero on days 40 and 41. Cumulative behavioral scores on days 14, 25, 41, and 55 calculated for each mouse are presented in Fig. 2.1B. PLP+OGF mice had a 66.7% decrease in the overall median cumulative score relative to that calculated for PLP+Saline mice over the 55- day experimental
Disease severity was determined for each mouse by calculating the number of days an individual mouse had a disease score greater than or equal to 4 divided by 55 (total days of study) (Fig. 2.1C). For approximately 40% of the time, PLP+Saline mice had behavioral scores ≥ 4 in comparison to PLP+OGF mice, whereas mice injected with OGF had severe disease scores for only 13% of the time. Combination of data from 2 experiments demonstrated marked reductions in median behavioral scores of OGF-treated mice relative to saline-treated mice with RR-EAE beginning on day 17 and continuing daily (except for day 35) for 40 days (Fig. 2.1D).

Relapse and Remitting Behavior

Periods of relapse and remission were calculated in a post-hoc manner for an individual animal. A mouse was considered to be in a relapse when its behavioral score for 2 consecutive days was 2 points higher than its average behavioral score for all 55 days. PLP+Saline mice exhibited 4.5-fold more relapses that PLP+OGF mice, having an average of 1.8 ± 0.2 relapses per mouse during the 55 day period while PLP+OGF mice had 0.4 ± 0.2 relapses (Fig. 2.2A). Seven mice receiving OGF never had a relapse, four mice had only 1 relapse, and 2 mice displayed 2 relapses. The length of time that mice spent in the first relapse was 6-fold longer for PLP+Saline mice than that for mice receiving OGF (Fig. 2.2B). Saline-treated EAE mice spent an average of 12 of the 36 days (time after first flare) in relapse whereas PLP+OGF mice had less than 2 days in relapse during the 36 day period following the first flare. The length of time that the first relapse lasted was also reduced for the OGF treated mice. PLP+Saline mice displayed a first relapse that extended for 6.4 ± 2.1 days, representing 37 ± 13% of their time in
relapse in comparison to the PLP+OGF mice with relapses that lasted only 2 days or approximately 7% of the observation period (Fig. 2.2C).

Remissions were calculated for 36 days following the initial flare. Mice were considered to be in remission when their individual behavioral scores returned to 0 or 0.5 for two consecutive days. Only one mouse in the PLP+Saline group exhibited a remission following the period of peak disease, whereas 9 of 13 PLP+OGF mice had remissions (Fig. 2.3A). Two of the remaining four PLP+OGF mice had behavioral scores that reached 0 or 0.5 but were not sustained for 2 consecutive days. Fifty-four percent of the OGF-treated mice returned to scores of 0 for greater than 2 consecutive days during the observation period. Sixty-two percent of the PLP+OGF mice had a remission within 14 days of disease onset in comparison to only 1 mouse in the PLP+Saline group displaying remission within 2 weeks. The only remission for the saline-treated mouse occurred after 9 days, whereas some OGF-subjected mice had remissions within 4 days (Fig. 2.3A). The cumulative time spent in remission was 16.5 ± 4 days for PLP+OGF mice while the PLP+Saline mouse was in remission for 3 days. Because only one saline treated mouse had a period of remission, the values could not be analyzed. However, the mean length of remission for PLP+OGF mice was 23.9 ± 3.7 days in comparison to a 14 day remission for the one saline-treated mouse (Fig. 2.3B). The latency time to the first remission was 6.2 ± 0.5 days for the PLP+OGF mice, while the latency for the only saline treated mouse to demonstrate a remission was 8 days.
Body weights were recorded over the course of the observations. Both PLP+OGF and PLP+Saline groups showed a significant drop in body weight from normal mice following immunization that extended for 3 weeks (Fig. 2.3C). However, body weights for the PLP+OGF mice increased beginning in the 4th week following PLP immunization, and were comparable with Normal mice for the remainder of the observation period (Day 27-55). PLP+Saline mice weighed significantly less than Normals on days 13, 20, 27, 41, 48, and 55, and were significantly lighter in weight than PLP+OGF mice on days 13, 41 and 55 (Fig. 2.3C).

Neuropathology

**Proliferation of Microglia and T lymphocytes**

Microglia/macrophages and lymphocytes are two cell types that proliferate in peripheral tissues in early response to immunization and then migrate to central nervous tissue. Lumbar spinal cord tissues from mice in each group were stained with antibodies to iba-1 to identify microglia/macrophages (Fig. 2.4A,C) and CD3 to identify T lymphocytes (Fig. 2.4B,D). Within 10 days of immunization with PLP\textsubscript{139-151} the number of Iba1+ cells was significantly increased in the spinal cord tissues of both groups. On day 14, the number of Iba-1 positive cells in the PLP-Saline group were increased 7-fold over normal levels, but were only elevated 4-fold in the PLP+OGF group. Normal mice without any immune reaction had relatively few (8-10 cells) Iba-1-positive cells. By day 55, the number of microglia/macrophages identified in the spinal cord was reduced by 50% in both groups for measurements on day 14; OGF treatment significantly repressed the number of Iba-1 positive cells relative to the number of cells in the PLP+Saline group.
Determination of the number of T lymphocytes was based on CD3$^+$ staining of spinal cord tissue on days 10, 14, and 55 (Fig. 2.4B). Prior to signs of clinical disease, the number of T lymphocytes, identified as small, round cells (inset) was elevated 2.4-fold in the PLP+Saline group relative to Normals, and the number of T lymphocytes in the PLP+OGF group was markedly reduced by 44% from that in the PLP+Saline group. At the peak of disease (day 14), PLP+Saline mice had lymphocytic proliferation that reached 34-fold more T lymphocytes than in normal mice. The number of T-cells in the PLP+OGF mice was reduced 2.4-fold from PLP+Saline levels, and was approximately 10-fold more than Normal values. At 55 days, the mean number of T lymphocytes was 182 cells/field for PLP+Saline mice in comparison to 95 cells/field for the PLP+OGF group, a 48% reduction in lymphocyte proliferation; Normal mice displayed 10 CD3$^+$ cells/field (Fig. 2.4D).

**Activated Astrocytes**

The number of activated astrocytes recorded in regions on either side of the central canal in the lumbar spinal cord after 10, 14, and 55 days of treatment is presented in Figures 2.5A, B. Prior to any appearance of clinical disease on day 10, the number of astrocytes were elevated in both PLP+Saline and PLP+OGF groups with a 3-fold or greater increase over baseline (7 astrocytes/field). At the time of peak disease (i.e., day 14), mice in the PLP+OGF groups had nearly 50% fewer activated astrocytes relative to PLP+Saline mice (57.1 ± 8.5 astrocytes/field) (Fig. 2.5B). On day 55, the PLP+Saline mice had 2-fold more activated astrocytes relative to PLP+OGF (33.7±4.4 astrocytes/field); PLP+OGF mice had comparable levels of astrocytes in their spinal
cords as Normals (Fig. 2.5B). Astrocytes are not activated and proliferating in non-immunized mice.

**Demyelination and Neuronal Damage**

Cross-sections of lumbar spinal cord tissue stained with Luxol fast blue were evaluated for areas of demyelination; no demyelinated regions were detected on day 10. Over the course of 55 days, less than 10% of the cross-sectional area of lumbar spinal cord displayed demyelination at any time (Fig. 2.6A). On days 14 and 55, the spinal cord sections from PLP+Saline mice had 5% and 7% demyelination, respectively, in comparison to approximately 2% of the cross-sectional area in PLP+OGF tissues at both time points, thus demonstrating no advancement of demyelination following the initial flare of clinical disease.

Neuronal damage in the ventral horn using cresyl violet stained sections revealed ~38% of the neurons were damaged on day 14 in both the PLP+Saline and PLP+OGF groups. After 55 days of treatment, PLP+Saline mice had nearly 70% damaged neurons in spinal cord specimens. Mice treated with OGF had 44% fewer damaged neurons on day 55 than observed in sections from PLP+Saline mice (Fig. 2.6B), with the overall percentage of damaged neurons remaining comparable to levels reported on day 14.

**Mechanism of Action for OGF**

Cell number within a tissue is reflective of alterations in proliferation, migration or cell death. To assess whether OGF treatment of mice with EAE affected cell proliferation, tissue specimens were stained with Ki67 to distinguish all cells recruited in
the active phases of the cell cycle (Fig. 2.7A). Analyses of Ki67+ cells in tissues from RR-EAE mice treated for 14 days revealed that normal mice had indices of 12.4 ± 2.3 percent proliferation whereas both PLP groups recorded twice the level (~25% proliferation) (Fig. 2.7B). By day 55 post immunization, proliferation was approximately 7% in normal animals and 20% in PLP+Saline mice. Analyses of spinal cord tissues from animals in the PLP+OGF group revealed 13.8 ± 1.9% dividing cells per field, a 32% reduction (p<0.01) from PLP+Saline mice.

Evaluation of images from serial sections stained for astrocytes and dividing cells revealed that the number of both GFAP+ and Ki67+ cells in PLP+OGF mice was reduced ~45% from that recorded in sections from PLP+Saline animals (~15%) (Fig. 2.7C).
Discussion

Relapse-remitting multiple sclerosis is the most common form of this autoimmune-related disorder and presents in patients as a wide spectrum of behavioral and pathological signs.\textsuperscript{1,24} This study demonstrates for the first time data that exogenous OGF treatment can prevent or mitigate relapses in a mouse model of RR-EAE when OGF treatment is initiated at the time of disease induction. OGF treatment initiated at the time of disease induction was effective at improving clinical signs during the initial flair and at preventing further relapses in mice. These observations were observed in separate experiments supporting the efficacy of OGF, as well as documenting the consistency of the PLP-induced model of RR-EAE.\textsuperscript{4} OGF treatment led to prevention of behavioral relapse for more than 36 days following the initial flair, with 85% of the mice returning to behavioral scores of 0 or 0.5 over the course of 5.5 weeks, and more than 70% of the mice showing remissions for more than 2 days. However, OGF administration at this dosage did not prevent the disease, nor did it “cure” the disease completely in any mouse. PLP+OGF animals displayed little more than limp tails and wobbly gait throughout a 36 day period following the first flair in comparison to the PLP+Saline group that experienced up to 4 relapses involving paralysis of one or more hindlimbs during the 8 week observation period.

This study confirmed and extended the use of a novel scoring system for behavior that is predicated on very detailed observations of motor and sensory responses in mice. Because changes in behavior are the basis for assessment of progression through distinct phases of the disease such as relapse and remission, the
evaluation of the capability of mice to perform complicated motor tasks such as righting reflexes and suspension from inverted mesh grids provided a broader assessment of clinical behavior. As with most disease models, mouse variability was evident. However, by conducting multiple experiments, the reproducibility of the disease model, as well as the effectiveness of OGF, were validated.

The PLP model of RR-EAE presented with a consistent level of spinal cord pathology. Mice immunized with PLP$_{139-151}$ and treated with saline expressed elevated numbers of microglia and macrophages in lumbar spinal cord tissue as detected by iba-1 staining within 10 days of immunization, and were 8-fold greater than the quiescent levels in normal, non-immunized mice at the time of peak disease on day 14. OGF treatment reduced proliferation or migration of microglia/macrophages into spinal cord tissue at 14 and 55 days. Likewise, OGF treatment markedly reduced the number of T-lymphocytes infiltrating spinal cord tissue on days 14 and 55 following immunization. RR-EAE presented in the spinal cord with increased numbers of activated astrocytes as recorded by GFAP positive staining as early as day 10, a time prior to the onset of behavioral signs of the disease. The numbers of activated astrocytes were diminished by OGF treatment at 14 and 55 days and were relatively constant throughout the observation period. Although oxidative stress was not measured in these studies, previous work$^{25}$ demonstrated that OGF suppressed overall nitric oxide synthesis by depletion of astrocyte cell number. Demyelination, a hallmark of multiple sclerosis and EAE, was also evident in mice with RR-EAE within 14 days of immunization.
The present study demonstrates that the endogenous peptide OGF is effective at preventing relapses in the mouse model of RR-EAE. These data confirm and extend earlier work demonstrating that OGF was effective against progressive EAE induced by immunization of mice with MOG_{35-55}. Daily administration of OGF or modulation of the OGF-OGFr axis by intermittent opioid receptor blockade with low dosages of naltrexone beginning at the time of EAE induction were effective at repressing the clinical signs of EAE and progression of disease. When OGF treatment was initiated at the time of immunization with MOG_{35-55}, some mice in the group never developed clinical signs of disease; all OGF-treated animals with EAE disease had diminished severity of behavioral deficits relative to EAE mice receiving saline. OGF treatment from the time of induction of disease also resulted in 6-fold more mice showing behavioral remission in comparison to animals receiving saline.

OGF reduction of T lymphocyte number within 14 days of treatment suggests that OGF mediates proliferating immune cells, and this could be a secondary pathway for modulation of autoimmune diseases. Reduction in specific T cell populations are the topic of further study, but several reports have indicated that OGF inhibits stimulated T cells in culture in a receptor-mediated manner^{12} and in mice immunized with MOG_{35-55} that produces chronic EAE in C57BL/6 mice^{8,10}. Other studies have shown that OGF inhibits proliferation and function of B cells isolated from normal murine spleens in vitro^{13} further supporting the role of endogenous opioids to repress immune-responsive cells, and thus diminish early phases of inflammation which often cause the behavioral signs observed by patients first suspecting MS^{26}. CD3+ staining for T cells did not
distinguish whether OGF was targeting CD4$^+$ or CD8$^+$ cells, and further studies are required to delineate specific immune-cell populations targeted by endogenous opioids.

The mechanism of OGF’s action in RR-EAE is not completely understood, and more studies are required to understand how the OGF-OGFr axis interacts with this autoimmune disease. However, OGF is a well-established inhibitory growth factor, with action that is mediated by the OGFr.\textsuperscript{5,6,27} OGF is neuropeptide that is autocrine and paracrine produced, and enters cells through active transport pathways using clathrin-mediated endocytosis\textsuperscript{28}, and targets cyclin-dependent inhibitory kinases, particularly p16 and p21 to retard the cell cycle.\textsuperscript{11}

Interestingly, both endogenous and exogenous OGF, as well as the precursor proenkephalin, is quickly degraded by enkephalinases.\textsuperscript{29-21} Several metalloproteinases (e.g., MMP-12, MMP-3, TIMP-1), required for proper conduction of the central nervous system activity, are upregulated in models of demyelination.\textsuperscript{32} One endopeptide in particular, neprilysin, is activated in patients with MS.\textsuperscript{33,34} Whether the rapid turnover of this inhibitory peptide plays a role in the establishment and/or progression of RR-EAE or RR-MS is unknown, but this information suggests that levels of endopeptidases should be monitored in patients to determine levels of OGF and enkephalinases during periods of relapse and remission.

Clinical trials with OGF and low dosages of naltrexone (LDN), which upregulate endogenous production of OGF, also need to be mindful of OGF biochemistry. To date, therapeutic treatments have targeted steroidal pathways.\textsuperscript{35-37} At the present time only a few, limited clinical trials on the use of OGF or LDN as therapies for MS have been
conducted;\textsuperscript{38,39} both studies reported improved quality of life in patients with this life-long neurological disorder. The present data, along with information on the efficacy of OGF or LDN for treatment of the chronic progressive model of EAE, warrant further randomized, controlled clinical trials in order to provide these compounds to patients with relapse-remitting or progressive MS.
Figures and Legends

A

Day Post-Disease Induction

Behavioral Score

PLP + Saline
PLP + OGF

B

Sum of Scores

Day Post Induction

14 25 41 55

C

Disease Severity (% of Time)

PLP + Saline
PLP + OGF

D

Behavioral Score

Day Post-Disease Induction
**Figure 2.1.** OGF treatment reduces severity of clinical signs of relapse-remitting EAE.

(A) Behavioral profile of RR-EAE from 18 mice immunized with PLP and treated daily with either 10 mg/kg OGF (PLP+OGF) or sterile saline (PLP+Saline) beginning at the time of immunization. Behavior was scored daily on all mice for 55 days. (B) Cumulative behavioral scores on days 14, 25, 41 and 55. (C) Disease severity represented as the percentage of time (number of days) that an animal had a disease score ≥ 4 during the 55 day period. (D) Combined behavioral scores from 2 separate experiments for 31 mice with RR-EAE treated with OGF (n=18) or saline (n=13). Values in A, B, and D are the median with interquartile ranges. Values in C represent means ± SEM. Significantly different from PLP+Saline group at p<0.05 (*), p<0.01 (**), or p<0.001 (***).
Figure 2.2. Treatment of RR-EAE mice with OGF reduced the number and length of relapses.

(A) Mean number of relapses during the 36 day period following the initial flair of disease. (B) Duration (days) of first relapse. (C) Cumulative length (days) of time each mouse was in relapse during the 55 day observation period. Values represent means ± SEM. Significantly different from PLP+Saline group at p<0.05 (*) and p<0.01 (**).
Figure 2.3. Treatment of RR-EAE mice with OGF increased time in remission and protected against weight loss. (A) Length of time for the first remission for PLP-immunized mice treated with either OGF or Saline. (B) Latency time (days) until the first remission. (C) Body weights of mice immunized with PLP and treated with OGF or saline, as well as normal unimmunized mice, during the experimental period. Values represent means ± SEM. Significantly different from Normal mice at p<0.05 (+) and p<0.01 (++); PLP+OGF differed from PLP+Saline mice at p<0.05 (*) on days 13, 41, and 55.
A 55 Treatment Days

Normal PLP + Saline PLP + OGF

Iba-1

CD3

C

D

Days Post Induction

Days Post Induction

Iba-1 Positive Cells / Field

CD3+ Cells / Field
**Figure 2.4.** The effect of OGF treatment on macrophage/microglia and T lymphocyte infiltration into the spinal cord of mice immunized with PLP and injected daily with 10 mg/kg OGF (PLP+OGF) or sterile saline (PLP+Saline) beginning at the time of disease induction. (A) Iba1 staining, with DAPI counterstain, of lumbar spinal cord sections from Normal mice and mice with RR-EAE at day 55. Control sections (inset in the normal panel) were stained with secondary antibody only. Scale bar = 50 μm. (B) Photomicrographs of CD3$^+$ staining, with DAPI counterstain, of central ventral regions of lumbar spinal cord sections from PLP-immunized mice treated with OGF or sterile saline for 55 days. Images from Normal mice were included for comparison. A CD3$^+$ cell is enlarged in the PLP+OGF panel inset. Control sections (inset in the Normal panel) were stained with secondary antibody only. Scale bar = 50 μm. (C) Number of iba-1 positive cells on either side of the central canal in lumbar spinal cord tissue after 10, 14, and 55 days of treatment with either OGF or sterile saline. (D) Number of CD3$^+$ cells/field after 10, 14, and 55 days of treatment, counted from the central ventral region of lumbar spinal cord sections (2-4 sections/mouse). Values in (C) and (D) represent means ± SEM. Significantly different from PLP+Saline mice at p<0.01 (**) or p<0.001 (***) . Significantly different from Normal mice at p<0.05 (+), p<0.01 (++) and p<0.001 (++++).
A 55 Treatment Days

Normal
PLP + Saline
PLP + OGF

B

Days Post Induction

0 10 14 55

Activated Astrocytes / 300μm²

Normal
PLP + Saline
PLP + OGF
**Figure 2.5.** The effect of OGF on astrocytes in spinal cord tissue of mice immunized with PLP and treated with OGF or saline. (A) GFAP staining of lumbar spinal cord sections from Normal mice and RR-EAE mice receiving either saline (PLP+Saline) or 10 mg/kg OGF (PLP+OGF) for 55 days. Control sections (inset) were stained with secondary antibody only. Scale bar = 50 μm. (B) Histograms represent the mean number (± SEM) of GFAP positive astrocytes per field in normal, unimmunized mice (Normal) and in mice receiving PLP and either 10 mg/kg OGF (PLP+OGF) or saline (PLP+Saline) beginning at the time of disease induction and treated for 10, 14, or 55 days. Significantly different from PLP+Saline mice at p<0.05 (*) and p<0.001 (***) and significantly different from Normal specimens at p<0.01 (++) and p<0.001 (+++).
Figure 2.6. Demyelination and neuronal damage is reduced in OGF-treated mice with RR-EAE. (A) The percentage of demyelination in white matter within the lumbar spinal cord on days 14 and 55 following immunization and treatment with either OGF (PLP+OGF) or saline (PLP+Saline). Values represent mean area ± SEM. (B) Histograms representing the percent (± SEM) of damaged neurons quantitated in cresyl violet stained sections of lumbar spinal cord relative to levels recorded for normal mice. Significantly different from PLP+Saline values at p<0.01 (**).
Figure 2.7. Reductions in cell proliferation of total cells and astrocytes in the spinal cord following OGF treatment of mice with RR-EAE. (A) Lumbar spinal cord sections from Normal mice as well as those immunized with PLP and receiving daily injections of sterile saline (PLP+Saline) or 10 mg/kg OGF (PLP+OGF) stained with Ki67. Control specimens were stained with secondary antibody only (inset); scale bar = 50 μm. (B) Ki67 labeling indices (mean percentage ± SEM) in the central ventral region of the lumbar spinal cord after 14 and 55 days of OGF treatment and RR-EAE. (C) Histograms (mean percentage ± SEM) of GFAP+ and Ki67+ cells in the ventral white matter of spinal cord tissue isolated from RR-EAE mice treated with OGF (PLP+OGF) or sterile saline (PLP+Saline) on day 14. Data in (B) and (C) represent means ± SEM; significantly different from PLP+Saline group at p<0.05 (*) and p<0.01 (**), and significantly different from Normal at p<0.05 (+) and p<0.001 (++++).
References

   http://www.nationalmssociety.org/about-the-society/ms-prevalence/index.aspx


CHAPTER 3. OGF TREATMENT OF ESTABLISHED RELAPSE-REMITTING EAE
Rationale

The first aim of this thesis demonstrated that OGF, when administered from time of disease induction, attenuates the course of RR-EAE. However, this is not clinically relevant, as patients are unlikely to see a physician prior to the presentation of symptoms. The goal of this aim is to determine whether OGF treatment attenuates the behavioral course of RR-EAE when administered at time of disease onset, and to determine the therapeutic effects of OGF on the histopathology associated with established RR-EAE.
Improved Clinical Behavior of Established Relapsing-Remitting Experimental Autoimmune Encephalomyelitis Following Treatment with Endogenous Opioids: Implications for the Treatment of Multiple Sclerosis

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Abstract

Relapse-remitting multiple sclerosis is a chronic disease of the CNS that affects 350,000 individuals in the U.S., reducing the quality of life and often resulting in paralysis. Most current therapies do not target the underlying pathophysiology of multiple sclerosis (MS). This study examined the therapeutic efficacy of an endogenous peptide (opioid growth factor, OGF) known to inhibit cell replication in a receptor-mediated manner, utilizing a mouse model of relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE). RR-EAE was induced by immunization of SJL/J mice with proteolipid protein. Two days following establishment of clinical disease, treatment with OGF (10 mg/kg) or saline was initiated and mice were observed on a daily basis. OGF treated mice had markedly reduced clinical signs of disease over the course of 40 days. OGF treatment increased the incidence and lengthened the time of remissions relative to saline-treated mice with RR-EAE. OGF therapy also reduced relapses, and facilitated extended periods of mild disease. Neuropathological examination of lumbar spinal cord after 40 days of treatment revealed decreased numbers of Iba-1 and CD3+ reactive cells, suggesting that OGF inhibited proliferation of microglia/macrophages and T lymphocytes, as well as decreasing the number of proliferating activated astrocytes (Ki67 and GFAP dual labeled sections). Peptide treatment for 40 days diminished levels of demyelination in comparison to saline-treated mice with RR-EAE. These data are the first to demonstrate that exposure to OGF initiated at the time of established disease can reverse the course of RR-EAE and reduce neuropathological deficits.
Introduction

Relapse-remitting multiple sclerosis (RR-MS) affects about 350,000 people in the United States, and more than 2.5 million individuals worldwide.\textsuperscript{1,2} The disease is manifested by proliferation and activation of T-lymphocytes, microglia, and astrocytes in the spinal cord, resulting in inflammation, demyelination, and axonal damage. As the disease progresses, neurodegeneration in the spinal cord and brain is prominent leading to a significant reduction in the quality of life. Current therapies are not completely effective and are designed to target one or more of the symptoms of the disease, rather than targeting disease-based mechanisms.\textsuperscript{3-5} The mouse model of relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) induced by proteolipid protein immunizations represents an animal model to study the clinical course of behavior and neuropathology in RR-EAE.\textsuperscript{6,7}

The biological pathway involving endogenous opioids, specifically opioid growth factor (OGF) and its receptor (OGFr), has been shown to mediate the course of progressive EAE.\textsuperscript{8,9} The OGF-OGFr axis can be modulated by either exogenous treatment with OGF, or by upregulation of OGF and OGFr following low dosages of naltrexone (LDN).\textsuperscript{8-11} Previous studies have reported that mice immunized with myelin oligodendrocyte glycoprotein (MOG) to establish progressive EAE and injected daily with 10 mg/kg OGF 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.\textsuperscript{8,9,12,13} Neuropathology of the lumbar spinal cord revealed significant reductions in the number of activated astrocytes and
regions of demyelination. Mice with established progressive EAE responded to exogenous OGF within 6 days, and the course of disease was reversed. Clinical signs of disease were markedly reduced from mice with EAE receiving saline, and spinal cord pathology was diminished. Animals with established chronic EAE receiving OGF had a reduced number of activated astrocytes and damaged neurons, decreased areas of demyelination, and repressed T cell proliferation.

Approximately 85% of patients initially present with relapse-remitting forms of MS. The mouse model for RR-EAE is distinct from the MOG-injected model of chronic progressive EAE and utilizes immunization of SJL/J mice with proteolipid protein (PLP). Within 9 days of treatment with OGF the RR-EAE animals had reductions in clinical signs of disease, with up to a 66% decrease in median cumulative disease scores relative to saline-treated RR-EAE mice. Importantly, no further remissions were noted over the course of 55 days. Assessment of the lumbar spinal cord revealed marked reductions in the number of T lymphocytes, microglia, and activated astrocytes.

The present study examined the effects of OGF for treatment of established RR-EAE by beginning OGF treatment 2 days after initial clinical signs of disease. The animals were observed daily for 40 days to evaluate relapses and remissions. Lumbar spinal cord tissue was examined histologically to assess the expression of proliferating neurons and glia.
Materials and Methods

Animals

Female 6-8 week old SJL/JORlCRL mice (Charles River Labs, Wilmington, MA) were housed 5 per cage under standard conditions in a room separate from other rodents, and acclimated for one week prior to immunization; food and sterile water were available ad libitum. Throughout the experiment, soft food and HydroGel (ClearH2O, Portland, ME) were placed on the floor of the cages as needed. All experiments were conducted in accordance with the National Institute of Health guidelines on animal care, and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Induction of RR-EAE

Animals were immunized with subcutaneous injections of 100 µg of myelin proteolipid protein 139-151 (PLP139-151) (Peptides International, Louisville, KY) following established protocols.\(^{14-16}\) PLP\(_{139-151}\) was prepared by dissolving 100 µg in 0.15 ml sterile phosphate buffered saline (PBS) and emulsifying with 0.15 ml incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO) plus 250 µg mycobacterium tuberculosis (H37RA, Difco Laboratories, Detroit, MI). The final mixture containing equal volumes of the phosphate-buffered saline (PBS) containing PLP\(_{139-151}\) and Freund's adjuvant was emulsified by vortexing. Mice received three 100 µl (total volume of 300 µl) injections subcutaneously on their back. Mice also received intraperitoneal (i.p.) injections of 200 ng pertussis toxin (List Biological Laboratories, INC., Campbell, CA) on days 0 and 2 post immunization. Mice were lightly anesthetized with 3%
isoflurane (Vedco, Inc., St. Joseph, MO) for PLP$_{139-151}$ and *M. tuberculosis* injections; anesthesia was not required for daily treatments.

**Drug treatments**

Mice immunized with PLP$_{139-151}$ were randomly assigned to treatment groups beginning on the second consecutive day of clinical disease score (i.e., established disease). Animals received i.p. injections (0.1 ml) of either 10 mg/kg OGF (Polypeptide Laboratories, Torrance, CA or Sigma-Aldrich, Indianapolis, IN) (RR-EAE+OGF) or sterile saline (RR-EAE+Saline). Treatments were administered daily between 1000 and 1100 hr throughout the study. Weights were monitored weekly.

**Behavior Assessment**

Two individuals observed mice daily beginning 8 days post-immunization, one observer was masked to the treatment groups. Disease scores were recorded daily using a 10-point scale.$^{13,14}$ Mice were placed on a smooth flat surface to observe tail tonicity, gait, and righting reflex. Individual limb strength was examined by placing mice inverted on a wire grid for no longer than 20 seconds. Each category is scored separately then summed with the others to reach a score between 0-10, where 0 = normal and 10 = death.

Disease onset was considered the second consecutive day the animal had a disease score of 0.5 or greater. Cumulative disease is the summation of behavioral scores over the 40-day treatment period. Complete remission was considered a return to a behavioral score of 0.5 or less for 2 consecutive days. Relapses were considered for an individual mouse when the behavioral score increased by at least 2 points for 2
consecutive days. Behavioral scores for mice that were euthanized or died were recorded but not included in the behavioral assessment.

**Neuropathology**

At designated time points (5, 14, and 40 days of treatment), mice were 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 intraventricular perfusion with fresh 4% paraformaldehyde (PFA). Vertebral columns were dissected and post-fixed in 4% PFA for 18 hours; spinal cords were removed and the lumbar enlargement was collected for analysis. The lumbar region was divided in half and processed for either paraffin embedding (caudal portion for demyelination) or frozen at -80°C (cranial portion). Regions of demyelination were assessed in sections stained with Luxol fast blue and neutral red. Frozen spinal cords were cryosectioned (10 µm) and stained for microglia/macrophages (1:200 iba-1, Wako, Osaka, Japan), T-lymphocytes (1:200 CD3, ab5690, Abcam, Cambridge, MA), activated astrocytes (1:300 GFAP, Cell Signaling, MA) and proliferation (1:200 Ki-67, ab66155, Abcam). Controls were stained with secondary only (1:1000 TRITC or FITC and 1:5000 DAPI). All imaging and analysis was performed using previously published protocols.\textsuperscript{13,14}

**Statistical Analysis**

Behavioral data over time were analyzed with a two-way analysis of variance (ANOVA) followed by post-hoc comparisons using Bonferroni multiple comparison test. Data on remissions and relapses were evaluated with non-parametric Mann-Whitney U tests. Morphological data were evaluated by student’s two-tailed t-tests or one-way
analysis of variance with post-hoc comparisons made using Newman-Keuls tests. All data analyses were performed with GraphPad Prism software (La Jolla, CA). \( P \) values of 0.05 or less were considered significant.
Results

General Observations

Fifty-five female SJL/J mice were immunized with PLP\textsubscript{139-151}. All mice were monitored daily for behavioral signs of disease, and after 2 consecutive days of clinical disease, mice were randomly assigned to groups receiving either 10 mg/kg OGF (RR-EAE+OGF) or 0.1 ml sterile PBS (RR-EAE+Saline). One mouse died prior to treatment. Twelve additional mice (7 saline, 5 OGF) died between days 10 and 13 post immunization after being assigned to a treatment group. The remaining 39 mice were monitored daily for 40 days of treatment.

OGF Reduces Clinical Disease Scores in Established RR-EAE

Immunization with PLP\textsubscript{139-151} resulted in the first appearance of clinical signs of RR-EAE on day 9, with the average day of disease onset occurring on day 10. The latency time (days) to peak disease following initiation of treatment ranged between 2 and 3 days (i.e., days 13 and 14 post immunization). No differences were noted in time of peak disease between OGF and saline-treated mice. RR-EAE+OGF mice exhibited lower daily disease scores compared to RR-EAE+Saline mice beginning after 14 days of treatment (Figure 3.1A). OGF treatment also diminished the overall clinical disease (sum of scores) (Figure 3.1B), with a behavioral score of 114.6 ± 7.7 compared to 143.4 ± 9.2 for RR-EAE+Saline mice. To gain further insight into the behavioral aspects of this disease model, disease states were characterized according to levels of behavioral scores: peak (initial flare), severe (score ≥ 4), mild (score ≤ 2) disease, and complete remissions (score ≤ 0.5). Relapses were indicated by an increase in clinical score of 2
or more points for 2 consecutive days. Evaluation of the entire 40-day behavioral period revealed that RR-EAE+Saline mice were classified with a severe disease for 16.2 ± 3.0 days after the initial flair compared to RR-EAE+OGF mice who had a disease severity index of 10.3 ± 1.5 days (Figure 3.1C).

Post-hoc analysis of daily behavioral scores revealed that RR-EAE mice receiving OGF appeared to display two distinct behavioral patterns – those mice that responded well to OGF treatment (responders) and those that did not (non-responders). Based on disease index values (average disease score over the 40 days of treatment) and the point at which an animal could move from mild disease to moderate/severe disease (i.e., index value of 3), groups of responders (RR-EAE+OGF-R) and non-responders (RR-EAE+OGF-NR) were established and their behavioral patterns evaluated (Figure 3.2A). The mean disease score for mice who did not respond to OGF was 3.6 ± 0.1, comparable to that of the animals receiving saline (3.4 ± 0.2). Both groups differed significantly from mean disease scores for the animals responding to OGF (RR-EAE+OGF-R) with a score of 2.1 ± 0.2. The time period for mice with behavioral scores ≥ 4 revealed that mice responding to OGF had fewer days with severe disease (5.3 ± 1 days) relative to the amount of time with severe disease scores for animals in the RR-EAE+OGF-NR group (17.0 ± 1.8 days) and RR-EAE+Saline control group (16.2 ± 3 days) (Figure 3.2B). In view of the findings of Summers de Luca regarding establishment of RR-EAE in mice purchased from Charles River,7 the substrain differences may explain variations in response to OGF. Nonetheless,
inclusion and analyses of all mice in the group revealed that OGF markedly altered the course of RR-EAE.

**Relapse and Remitting Behavior**

**Remissions**

Relapses and remissions were calculated post-hoc for each animal, and recorded throughout the observation period. Mice were considered to be in complete remission when their individual behavioral scores returned to 0 or 0.5 for two consecutive days. Over the course of 40 treatment days, approximately 42 percent of mice in the RR-EAE+OGF group had at least one period of complete remission (score ≤ 0.5 for 2 consecutive days) in comparison to one of 13 mice in the RR-EAE+Saline group (Figure 3.3). The one RR-EAE+Saline mouse spent a total of 2 days in complete remission beginning 19 days after peak disease.

The length of time (days) that RR-EAE+Saline mice spent in complete remission was 0.1 ± 0.1 day because only one saline mouse had a complete remission. In comparison, the mean length of complete remission for OGF-treated mice was 3.4 ± 1.2 days (Figure 3.3A) beginning approximately 15.8 ± 2.9 days after peak disease (Figure 3.3A, C). Only one mouse in the saline group had a remission, on day 19 whereas 11 of 26 mice receiving OGF had complete remissions (Figure 3.3B). The latency between treatment initiation to the first complete remission (days) for each mouse revealed that the saline- treated mouse went into remission on day 19, whereas some of the OGF-treated mice had remissions during the first week of treatment (Figure 3.3C). All of the mice responding to OGF treatment had at least one remission during the 35 day period
of time following peak flair. For comparison, 10 of 14 mice in the group that responded
to OGF had complete remissions and 5 mice appeared to remain in permanent remission for extended periods of time.

In addition to periods of complete remission, mice treated with OGF expressed sustained intervals of mild disease activity, defined as days with behavioral scores less than or equal to 2 (Figure 3.3D). In comparison to saline-treated mice that had mean duration of 6.8 ± 2 days of mild disease activity, OGF treated mice had mild disease scores for 13.6 ± 2 days, and mice with RR-EAE responding to OGF had behavioral scores that were considered mild for 26.7±1.9 days. Of the 26 mice treated with OGF, 11 animals had one or more complete remissions; one mouse had 3 independent periods of behavioral scores ≤ 0.5 (i.e., complete remission). The mean number of remissions for the entire group is 0.5 ± 0.1 in comparison to less than one remission (0.07± 0.07) for the saline treated group, as only one mouse had one remission (Figure 3.3B).

Evaluation of the incidence and length of mild disease (score ≤ 2) revealed that approximately 85% of animals in the RR-EAE+OGF group had mild clinical disease scores, whereas approximately 38% of RR-EAE+Saline never demonstrated mild behavioral profiles. The period (days) of mild disease was significantly increased for OGF-treated mice (p = 0.044, non-parametric statistics), with OGF animals displaying a median of 16.5 days in comparison to RR-EAE + Saline (3 days) (Figure 3.3D). Of those RR-EAE+OGF mice that spent time in a mild clinical disease state, 62.64% remained in this phase of disease for longer than 13 days.
Relapses

A mouse was considered to relapse when the behavioral score increased by at least 2 points for 2 consecutive days (Figure 3.4). OGF treatment of RR-EAE reduced the length (Figure 3.4A) and number of relapses (Figure 3.4B) relative to RR-EAE animals treated with saline. Approximately 85% of RR-EAE+Saline mice had at least one relapse, and 23% of the group displayed 2 behavioral relapses over the 40-day observation period in comparison to 61.5% of RR-EAE+OGF mice. The mean length of relapse was 4.5 days for OGF treated animals in comparison to 9.2 days for saline treated mice (Figure 3.4A). Although the latency to relapse ranged between 15 and 22 days, the mean latency time from the start of treatment to the first relapse did not differ between the two groups (Figure 3.4C).

Environmental Stress: Housing

Housing and incidence of fighting did not appear to be a causative factor for any biphasic responses to OGF treatment. There was no correlation between mice that were observed to have minor or few bite wounds and clinical scores. Animals with severe tail wounds were euthanized immediately and their data removed from the study. Furthermore, there was no correlation or relationship between housing and response to OGF. Within a single cage, mice were randomized to receive either treatment and there was no correlation between assignment order and housing, with response to OGF therapy. Cage position was not a factor as mice housed in cages on the top rack or bottom rack, or inside or outside, were scored as responders and non-responders and represented both treatment groups.
The velocity to peak disease was evaluated by linear regression comparing mean behavioral scores for 7 days prior to peak disease. No differences were noted and there was no correlation between onset of disease and response to OGF.

**Neuropathology**

**OGF Plays a Role in Modulating Microglia/Macrophage Activity and CD3 Infiltrates After 40 Days of Treatment in Established RR-EAE**

To evaluate microglia/macrophages, lumbar spinal cord sections collected after 5 (acute disease), 14 (first relapse) and 40 (chronic disease) days of treatment were stained with an Iba1 antibody (Fig. 5A,C), and the number of positive cells recorded. Relative to non-immunized normal animals, the numbers of Iba-1 positive cells were increased 3- to 5-fold in all groups of RR-EAE mice. Within 5 days of established disease, the average numbers of microglia/macrophages per field were approximately 79 in saline-treated mice and 64 in OGF-treated mice. By day 40, OGF therapy reduced the number of positive cells to 35.9±4.8 in OGF-treated specimens relative to 108±14 positive cells in saline-treated RR-EAE mice, a reduction of nearly 67% (Figure 3.5C).

T lymphocyte infiltration was evaluated by staining lumbar spinal cord sections with a CD3 antibody (Figure 3.5B, D). CD3$^+$ cells were not observed in lumbar spinal cords on day 5. However, within 2 weeks of treatment, mice in the RR-EAE+Saline group had more than 100 T lymphocytes per field. No differences in the number of T-lymphocytes were noted between OGF and saline-treated mice on day 14, but by day 40, OGF therapy reduced CD3$^+$ T-lymphocyte infiltration by 57.4% from the average of
153 T-lymphocytes per field observed in lumbar spinal cord sections from RR-EAE+Saline mice (Figure 3.5D).

The Effects of OGF on Proliferation and Astrocytes

Ki67 was utilized to determine whether OGF inhibited total cell proliferation within the central ventral white matter of the lumbar spinal cord (Figure 3.6A, C). Analyses of spinal cord sections at 14 and 40 days post treatment revealed that OGF treatment significantly reduced cellular proliferation to less than 3 percent. In comparison, mice in the RR-EAE+Saline group demonstrated more than 7 and 10 percent proliferating cells after 14 and 40 days of treatment, respectively (Figure 3.6C).

Astrogliosis was measured by evaluation of the number of activated astrocytes in the central ventral white matter of the lumbar spinal cord (Figure 3.6B, D). Immunization with PLP_{139-151} markedly increased the number of astrocytes in the RR-EAE mice in comparison to normal mice. However, OGF exposure significantly reduced the number of GFAP^+ astrocytes on days 5, 14, and 40 relative to saline-treated controls (Figure 3.6D). To determine the percentage of activated astrocytes undergoing proliferation within the central ventral white matter of the lumbar spinal cord, sections were double-labeled with Ki67 and GFAP (Figure 3.6E, F). Examination of the dual labeling in lumbar spinal cord on days 14 and 40 showed that RR-EAE+OGF mice had a lower percentage of proliferating astrocytes (3.04 ± 0.76 and 2.29 ± 0.12) in comparison to RR-EAE+Saline animals (Figure 3.6F).
OGF Protects Against Demyelination in Established RR-EAE

Demyelination was evaluated by staining paraffin embedded lumbar spinal cord sections with Luxol fast blue and counterstained with neutral red (Figure 3.7A). Normal mice receiving saline at the time of immunization demonstrated negligible demyelination over the 40 day observation period. After 2 weeks of treatment, saline treated mice had approximately 25% of the spinal cord demyelinated in comparison to 13% for OGF-treated RR-EAE mice (data did not differ). However, after 40 days of treatment, the group of RR-EAE+OGF mice had approximately 8.7% demyelinated regions relative to more than 16% demyelination measured in spinal cords from RR-EAE+Saline mice (Figure 3.7B).
Discussion

Multiple sclerosis frequently begins with the relapse-remitting form that presents as a wide spectrum of behavioral signs and evolves over years to a more chronic, progressive form.\textsuperscript{1,2,17} This study demonstrates for the first time that exogenous treatment of OGF can reverse the pattern of relapses and promote a sustained period of remission in mice with established RR-EAE. OGF treatment initiated after observing clinical signs of disease effectively prevented further relapses in more than 38% of the mice receiving the endogenous opioid, and all OGF-treated mice had periods of mild disease in comparison to RR-EAE mice injected with saline.

The use of the detailed observations of both motor and sensory skills in mice allowed for assessment of progression through distinct phases of the disease such as relapse and remission. By incorporating multiple observers, with at least one masked to treatment, as well as conducting several studies, the RR-EAE model was validated as representative of RR-MS. Unlike treatment of RR-EAE at the time of immunization, OGF did not alter the severity of the initial flair (i.e., peak disease) as it occurred within 4-5 days following initiation of therapy. However, changes in the course of disease were detectable within 8 days of therapy.

Post-hoc evaluation of functional outcomes for OGF-treated animals revealed that some mice did not respond as well to treatment as other mice receiving OGF. These observations, along with correlation of mice and behavioral scores, developed a bimodal clinical behavioral profile as shown in Figure 3.2. This distinction was not evident in any spinal cord pathology, and most likely can be attributed to the genetic
background of SJL/J mice purchased from Charles River. In previous studies using Charles River SJL/J mice, treatment beginning at the time of immunization appeared to affect most mice by reversing the course of disease, reducing the severity of peak disease, and preventing further relapses. Utilizing the current treatment profile and definitions of “complete” remission whereby the mice had behavioral scores of 0.5 or less, the mice responding to OGF experienced up to 2 weeks of complete remission. This subgroup may provide an interesting study group as these mice have a different pathophysiological response to PLP immunization. Future investigations will be required to explore metabolic responses to OGF and other physiological pathways that may be dysregulated in RR-EAE. Stress related to housing or cage placement, as well as random assignment of treatment, revealed no correlation with behavioral response.

Morphologically, in the current study, mice receiving OGF had reduced proliferation of cells associated with disease progression such as astrocytes and CD3+ T cells. In some cases, cell numbers (i.e., total cells) were reduced within 2 weeks of OGF treatment suggesting that endogenous opioid exposure was able to inhibit replication of immune-related cells shortly after treatment began. Although reduction in astrocytes, glia/macrophages, and T lymphocyte number correlated with increased periods of remission, additional studies are required to assess specific immune cell migration into the spinal cord in these animals in order to develop a full spectrum of mechanism.

Earlier reports have shown that control mice immunized with PLP139-151 have elevated numbers of microglia and macrophages in lumbar spinal cord tissue, as well as
increased activated astrocytes.\textsuperscript{14} OGF is an inhibitory pentapeptide that upregulates cyclin-dependent inhibitory kinases to retard the cell cycle.\textsuperscript{11} Specific kinases involved in OGF’s action are not fully understood for EAE, but Ki67 staining suggests an overall reduction in the number of T lymphocytes infiltrating spinal cord tissue, along with repression of activated astrocytes, appears to be involved in established RR-EAE, as well as the chronic progressive model.\textsuperscript{13} Specific mechanisms of action elicited by the OGF-OGFr axis on immune cell replication involved repression of DNA synthesis and cell cycling. \textit{In vitro} studies have demonstrated that astrocyte proliferation as well as T and B lymphocyte proliferation is directly inhibited by OGF exposure with inhibition being receptor-mediated by OGFr.\textsuperscript{18-20}

The present study demonstrates that the OGF-OGFr axis is intricately involved with progression of EAE, and presumably this translates to MS. Because it has been shown that LDN\textsuperscript{21} and OGF\textsuperscript{13} can change the course of established disease, it appears that the OGFr is intact and able to interact with peptide or antagonist; this interfacing results in inhibition of cell replication. Circulating levels of OGF in animals (and humans) may be insufficient to counter the inflammatory processes that follow immunization. Whether dysregulation of the inhibitory peptide OGF occurs prior to immunization or after, and details of the dysregulation will require further study. However, given that exogenous OGF treatments are effective for both progressive and relapse-remitting EAE\textsuperscript{8-10,13,14} suggests that the underlying pathophysiology may be the same and share similar manifestations. Furthermore, it is known that the peptidases that break down enkephalins, and specifically neprilysin, are activated in patients with
MS.\textsuperscript{22,23} Whether the dysregulation of the OGF-OGFr axis involves increased enkephalinase activity\textsuperscript{24-26}, as well as decreased processing of the precursor proenkephalin is unknown.

Other investigations\textsuperscript{27,28} have also observed that neurotransmitters share both immunomodulatory and neurotransmission functions. Phenelzine was more effective at reducing clinical signs of EAE when administered at the time of immunization.\textsuperscript{27} When administered to mice with established EAE, glial cell proliferation and CD4\textsuperscript{+} migration to the spinal cord were unaffected, but elevated levels of GABA and dopamine were observed in the ventral horn of the spinal cord. EAE mice also have been shown to have hypersensitive responses to tactile stimulation with heightened gene expression of relevant cytokines.\textsuperscript{28} The present data supports and extends previous basic research demonstrating the efficacy of OGF or LDN treatment for chronic progressive EAE, and OGF for relapse-remitting EAE. Clinical trials using OGF or LDN as therapy have reported improved quality of life in MS patients,\textsuperscript{29,30} and together, the data warrant further randomized, controlled clinical trials in order to gain approval of OGF and/or LDN as treatment for patients with relapse-remitting or progressive MS.
Figures and Legends

A

Days of Treatment

Mean Behavioral Score

RR-EAE + Saline
RR-EAE + OGF

B

Sum of Scores

RR-EAE + Saline
RR-EAE + OGF

C

Disease Severity (Days)

RR-EAE + Saline
RR-EAE + OGF
**Figure 3.1.** OGF treatment initiated at the time of established disease ameliorates the clinical behavior of SJL/J mice with RR-EAE. Daily injections (0.1 ml) of OGF (10 mg/kg; n = 26) or saline (n = 13) were initiated after 2 consecutive days of clinical behavior (day 0). (A) Behavior was scored daily by individuals masked to treatment and continued for 40 days. Values represent mean ± SEM. (B) Sum of daily disease scores over 40 treatment days were significantly decreased by treatment with OGF. (C) Disease severity was calculated as the number of days that animals presented with disease scores ≥ 4. Values represent mean ± SEM. Significantly different from saline-treated mice at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***)
**Figure 3.2.** Clinical disease scores and disease severity revealing two diverse responses to OGF in mice with EAE, mice that responded (R) and non-responders (NR).

(A) Individual mouse disease scores (average) for 40 days of treatment were distributed as responders (above the dotted line indicating behavior score of 3) or below as non-responders. (B) Disease severity (days) indicated as time with a clinical disease score of 4 or higher for mice receiving saline (RR-EAE+Saline, n = 13) or OGF (RR-EAE+OGF-R, n = 15 and RR-EAE+OGF-NR, n = 11). Data represent mean ± SEM. Significantly different from mice receiving saline at p < 0.001 (*** and p < 0.0001 (****); significantly different between OGF responders and non-responders at p < 0.001 (+++ or p < 0.0001 (++++).
**Figure 3.3.** OGF treatment increased length and frequency of remissions. (A) Length of time (days) spent in complete remission (behavioral scores \( \leq 0.5 \)) during the 40-day observation period. One of the 13 saline treated mice experienced a complete remission (score \( \leq 0.5 \) for 2 consecutive days after peak disease) compared to the 11 of 26 OGF treated mice. (B) Number of complete remissions during the 40-day observation period. (C) Latency time (days) between peak disease and onset of complete remission. (D) Length of time (days) each mouse expressed mild disease (behavioral score \( \leq 2 \)). Data were analyzed with Mann–Whitney U tests, and values expressed as median. Significantly different from the saline treated group at \( p < 0.05 \) (*).
Figure 3.4. OGF treatment results in diminished relapses. (A) Length of time the animal remained in a relapsed. (B) The number of relapses experienced was decreased within the RR-EAE+OGF group. (C) The length of time after peak disease to the next disease flare. All data represent mean ± SEM. Significantly different at $p < 0.05$ (*).
40 Treatment Days

A

Normal | RR-EAE + Saline | RR-EAE + OGF

Iba-1

B

CD3

C

Days of Treatment

D

Days of Treatment

No.

Normal | RR-EAE + Saline | RR-EAE + OGF

Iba-1 Positive Cells / Field

CD3+ Cells / Field

| 5 | 14 | 40 |

| 0 | 50 | 100 | 150 |

| 0 | 50 | 100 | 150 |

| 0 | 50 | 100 | 150 |

| 0 | 50 | 100 | 150 |
Figure 3.5. OGF 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 post-treatment. Lumbar spinal cord sections were stained with Iba-1 antibodies to detect microglia/macrophages (A) or CD3 antibodies to detect T lymphocytes (B); sections stained with secondary antibody are shown in the inset. Bar = 50 μm. (C,D) The number of positively stained cells was recorded in 5 fields/section of central ventral white matter (n = 6 mice per group). Values represent mean ± SEM. Significantly different from normal mice at p < 0.05 (+), p < 0.01 (++), p < 0.001 (+++), and p < 0.0001 (++++); significantly different from RR-EAE mice receiving saline at p < 0.01 (** and p < 0.0001 (***) .
**Figure 3.6.** OGF inhibits cellular proliferation (A) and activation of astrocytes (B,C) in lumbar spinal cord from mice with established RR-EAE. Mice were evaluated at 5, 14, and 40 days post-treatment (time of established clinical disease). Ki-67 stained (A) or GFAP-stained (B) specimens of lumbar spinal cord from normal and RR-EAE treated with OGF or saline at 40 days of treatment. Lumbar spinal cord sections from normal and RR-EAE treated with OGF or saline. (C) Ki-67 and GFAP double-labeled sections showing proliferating astrocytes. Sections stained with secondary antibodies only are shown in insets; bars = 50 μm. The number of positively stained cells was recorded in 5 fields/section from 6 mice per group at each time point. (C) Double labeling with Ki-67 and GFAP within the central ventral white matter shows a decrease in proliferating astrocytes with treatment of OGF. Inset of proliferating astrocyte, demarcated by the red projections (TRITC=GFAP) surrounding a green nucleus (FITC=Ki-67). Values represent mean ± SEM. Significantly different from normal mice at p < 0.01 (++), p < 0.001 (+++), and p < 0.0001 (++++); significantly different from RR-EAE + Saline mice at p < 0.05 (*), p < 0.01 (**) and p < 0.0001 (***)

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Figure 3.7. OGF limited the amount of demyelination within the lumbar region of the spinal cord. (A) Lumbar spinal cord sections were stained with Luxol fast blue and counterstained with neutral red. (B) Area of demyelination (percentage) for RR-EAE mice treated with OGF or saline on treatment days 14 and 40. Significantly different from normal at $p < 0.05$ (+), $p < 0.01$ (++) and $p < 0.0001$ (++++) and from RR-EAE + Saline at $p < 0.01$ (**).
References


CHAPTER 4. LOW DOSE NALTREXONE TREATMENT OF ESTABLISHED RELAPSE-REMITTING EAE
Rationale

The first two specific aims have demonstrated the positive effects of administering the endogenous opioid, OGF, in mice with RR-EAE when therapy begins at immunization and at time of clinic presentation. A rising trend in therapy utilized by multiple sclerosis patients is low dose naltrexone, which intermittently blocks the OGF-OGFr axis. While there have been a few small clinical studies investigating LDN as a potential MS approved therapy, larger scale clinical trials are required. Currently, it is unknown whether intermittent blockade of the OGF-OGFr axis with LDN will attenuate disease progression in established RR-EAE. The goal of this aim is to determine the effects of intermittent blockade of the OGF-OGFr axis through administration of LDN after two days of clinical disease through daily behavior assessments, and to determine the therapeutic effects of LDN on the histopathology associated with established RR-EAE.
Low Dose Naltrexone Treatment of Established Relapsing-Remitting Experimental Autoimmune Encephalomyelitis

Leslie A. Hammer, Ian S. Zagon, and Patricia J. McLaughlin

Abstract

Relapse-remitting multiple sclerosis is a chronic disorder that affects more than 350,000 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 model of relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE).

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

LDN treatment significantly reduced behavior scores across the 40 day observation period. Number of remissions were increased in LDN-treated groups relative to controls. A bimodal distribution of behavioral responses to LDN distinguished “responders” from mice considered “non-responders” that showed behavioral characteristics similar to saline-treated animals. More than 60% of the mice responding to LDN had several days of remission. LDN-treated mice also had reduced areas of demyelination and decreased numbers of macrophages/microglia and activated astrocytes, as well as reductions in spinal cord demyelination, relative to saline-treated controls.
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.
Introduction

More than 2.3 million individuals worldwide have multiple sclerosis,\textsuperscript{1,2} 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.\textsuperscript{1,2} Approved therapies often target symptoms of the disease rather than underlying mechanisms, and thus are not completely effective leading to reduced compliance.\textsuperscript{1-6}

A novel biological pathway has been identified that regulates homeostasis of replicating cells and tissues.\textsuperscript{7} This pathway involves the endogenous opioid termed methionine enkephalin (opioid growth factor, OGF) and its nuclear-associated receptor, OGF receptor (OGFr).\textsuperscript{7,8} Modulation of the pathway can be direct by exogenous administration of OGF peptide or indirectly by stimulating endogenous 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 has effectively inhibited cancer cell replication.\textsuperscript{9} LDN treatment changes the course of progressive experimental autoimmune encephalomyelitis (EAE).\textsuperscript{10,11} Previous studies have reported that C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG) 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.\textsuperscript{10-12} Neuropathology of the lumbar spinal cord revealed
significant reductions in the number of activated astrocytes and regions of demyelination.\textsuperscript{10,11}

Despite more than 85\% of patients presenting initially with relapse-remitting forms of MS,\textsuperscript{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.\textsuperscript{13} In general relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) is induced in SJL/J mice by immunization with proteolipid protein 139-151 (PLP\textsubscript{139-151}).\textsuperscript{14-17} The animal response is manifested by proliferation and activation of T-lymphocytes, microglia, and astrocytes, resulting in inflammation, demyelination, and axonal damage, giving rise to well-defined clinical behavior and neuropathology.\textsuperscript{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.\textsuperscript{13} Our laboratory has established this model of RR-EAE and initiated treatment at the time of induction before behavioral changes were observed.\textsuperscript{18} Daily treatment with exogenous OGF resulted in RR-EAE animals that had reductions in 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.\textsuperscript{18} Importantly, the number 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/J mice that responded to OGF, reduce relapses, and render many mice in total remission.\textsuperscript{19}

Finally, a few clinical trials have utilized LDN for treatment.\textsuperscript{20,21} Of interest, 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.\textsuperscript{22}

The present study examined the effects of LDN treatment on established RR-EAE disease beginning LDN treatment 2 days after initial clinical signs. The animals were treated and observed daily for 40 days to evaluate relapses and remissions. Periodically, lumbar spinal cord tissue was 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/JOrlCoCrl 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 Institute 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 a fragment of proteolipid protein 139-151 (PLP_{139-151}) in 80 SJL/J mice.\textsuperscript{14,15} Mice were inoculated subcutaneously on the back with multiple injections (total volume 300 μl) of an emulsion containing 100 μg PLP_{139-151} (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 PLP_{139-151} 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 PLP_{139-151} and *M. tuberculosis*, but was not used for daily therapeutic treatments. Ten mice were not immunized and served as normal controls for comparisons of spinal cord pathology.
Drug Treatments

Drug treatments were initiated at the time of established disease. Mice were observed daily for behavior and scored based on the criteria described below.\textsuperscript{18,19} Treatments were initiated when mice displayed characteristic signs of RR-EAE for 2 consecutive days. 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 hr, 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 (scores were reconciled at time of observations) by placing each animal on a smooth surface and recording tail tonicity, gait, and righting reflex. Limb strength was assessed by inverting the animal on a wire grid and observing the ability for each mouse to maintain their grasp. A modified behavioral scale of 0 to 10 was used in order to record incremental changes in behavioral events.\textsuperscript{18,19,23} Each mouse received scores for tail tonicity, gait, righting reflex, and individual limb tonicity, and the sum of scores 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,\textsuperscript{24-27} or those reported by
our group.\textsuperscript{18,19,23} 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 on either side of the central canal and had a nucleus (DAPI) surrounded by TRITC with at least one projection. Macrophages were identified within the central ventral white matter (CVWM) and had to have a DAPI stained nucleus surrounded by both TRITC and FITC. 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) staining enabled assessment of proliferating, activated astrocytes. T lymphocytes were quantified as total CD3$^+$ 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 (CVWM) at 20x magnification using SlideBook software (Intelligent Imaging Innovations, Ringsby, 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). 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 was analyzed using Mann-Whitney U tests; morphological data were analyzed using the 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 PLP_{139-151} 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 4.1A). 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 and saline-treated mice. The effects of LDN treatment on behavior are presented in Figure 4.1A and 4.1B. 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 4.1B); thus mice were re-evaluated as 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 4.1B 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 RR-EAE+Saline mice that displayed a clinical score (sum of behavioral scores) of 139.2 ± 11.5, RR-EAE+LDN-R mice had a clinical score of 81.0 ± 6.8 in
contrast to RR-EAE+LDN-NR animals that had a clinical score of 159.0 ± 9.1. Values for non-responders did not differ from saline-treated animals.

**Relapse and Remitting Behavior**

Relapses and remissions were calculated post-hoc for each animal, and scored throughout the observation period (Figure 4.2). All mice were combined for analyses of relapses and remissions. Mice were considered to be in complete remission when their individual behavioral scores returned to 0 or 0.5 for two consecutive days. Mean latency time to the first complete remission was 14 days for saline-treated mice and 13 days for mice receiving LDN. Only 3 mice in the RR-EAE+Saline group exhibited a remission following the period of peak disease, whereas 7 animals receiving LDN displayed at least one complete remission 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 ± 21.6 days for RR-EAE+LDN mice (Figure 4.2A).

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 4.2B). 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).

Relapses were scored when a mouse displayed a daily behavioral score increase by at least 2 points for 2 consecutive days (Figure 4.2C). 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 a strong 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 4.3A, C), or CD3 to assess the number of CD3+ T lymphocytes (Figure 4.3B, 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 disease, the average numbers of microglia/macrophages per field, 20x magnification, 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 cells in normal mice, 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 4.3D). However, within 2 weeks of established disease, mice in the RR-EAE+Saline group had approximately 114 lymphocytes per field in comparison to 61 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 4.3D).

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. 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 4.4).

**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 4.5A) 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 4.5C. 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 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 4.5B) demonstrated significant increases in the number of GFAP$^+$ astrocytes at 5, 14, and 40 days of
treatment (Figure 4.5D). 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 lumbar spinal cord sections and counting GFAP+Ki67+ cells in the central ventral white matter (Figure 4.5E, 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 4.5F).

**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 4.6A). Normal mice demonstrated negligible demyelination over the 40 day observation period. After 2 weeks treatment, saline treated mice had approximately 22% demyelination in comparison to less than 5% (p < 0.01) for RR-EAE+LDN mice (Figure 4.6B). 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.\textsuperscript{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 when treatment of RR-EAE begins at the time of immunization, LDN did not markedly reduce the severity of the initial flare 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/MS by demonstrating that LDN therapy, which indirectly upregulates production of OGF and OGFr, effectively repressed 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 PLP\textsubscript{139-151} revealed comparable patterns of behavior among the saline-treated RR-EAE groups.\textsuperscript{19}
With regard to pathobiology of the spinal cord, PLP$_{139-151}$ immunization resulted in elevated number of microglia and macrophages in lumbar spinal cord tissue, as well as increased activated astrocytes. Whether these findings 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 cells migration through gel matrices.$^{31}$

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 PLP$_{139-151}$ induction model of SJL/J mice in this study also resulted in a group of mice that did not respond to treatment. Whether there are other epigenetic factors involved in these biphasic responses is to date unknown, thus 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.\textsuperscript{33} The underlying evidence for inhibited cell replication is supported by our research on the chronic progressive model.\textsuperscript{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.\textsuperscript{34}

The present study demonstrates that the OGF-OGFr axis is intricately involved with progression of RR-EAE, and this may translate to patients with relapse-remitting MS. Given that both OGF, and now LDN, have been documented to change the course of disease, it would suggest that the OGFr 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,\textsuperscript{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.
Figures and Legends

A

B

Days of Treatment

Days of Treatment
**Figure 4.1.** Mean clinical disease scores of SJL/J mice with RR-EAE receiving LDN beginning at the time of established disease.  (A) Behavioral scores for all mice immunized with PLP and treated daily beginning after 2 consecutive days of behavior (day 0); treatments began approximately 10 days following immunization. Behavior was scored daily on all mice for 40 days after recording established disease. RR-EAE+Saline mice (n=25) were injected with 0.1 ml saline, whereas RR-EAE+LDN (n=23) treated animals received 0.1 mg/kg naltrexone.  (B) Post-hoc analyses of mean behavioral scores for mice revealed a biphasic response to LDN whereby 50% of the mice demonstrated markedly reduced clinical signs of disease, while the other 50% had behavioral scores comparable to saline-injected RR-EAE animals.  Values represent means ± SEM.  Significantly different behavioral scores between RR-EAE+Saline and RR-EAE+LDN-R mice at p<0.05 (*), p<0.01 (**), p<0.001 (***), and p<0.0001 (****).  Significant differences in behavioral scores between the two groups of mice receiving LDN are indicated by p<0.05 (+), p<0.01 (++), p<0.001 (+++), and p<0.0001 (++++).
Figure 4.2. 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 observation period. (B) Length of time (days) in mild disease (behavioral score ≤ 2). (C) The number of days spent in relapse. Data represent means ± SEM. Significantly different from mice RR-EAE+Saline at p< 0.05 (*).
14 Treatment Days

A

Iba-1

Normal  RR-EAE + Saline  RR-EAE + LDN

B

CD3

C

Iba-1 Positive cells/Field

Days of Treatment

D

CD3+ Cells/Field

Days of Treatment

* Normal  ** RR-EAE + Saline  *** RR-EAE + LDN
Figure 4.3. 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 on either side of the central canal after 5, 14 and 40 days of treatment. (D) The number of CD3+ cells were recorded in the central ventral white matter of spinal cord collected from mice treated with LDN or saline for 14 and 40 days. Values represent means ± SEM for 3-6 mice per group. Significantly different from normal mice at p<0.05 (+), p<0.01 (++), p<0.001 (+++); significantly different from RR-EAE mice receiving saline at p<0.05 (*) and p<0.01 (**).
**Figure 4.4.** LDN treatment inhibited macrophage infiltration into the spinal cord.

Lumbar spinal cord sections at 14 and 40 days of treatment were dual stained with antibodies to F4/80 and Iba-1. (A) Double-labeled cells were considered macrophages and counted in the central ventral white matter. Sections stained with secondary antibody only are shown in the inset. Bar = 50 µm. (B) The numbers of double-labeled cells were significantly increased in RR-EAE+Saline mice at both 14 and 40 days. LDN reduced macrophage infiltration relative to that recorded in saline-treated animals. Values represent means ± SEM for 3-6 mice per group. Significantly different from normal mice at p<0.001 (+++); significantly different from RR-EAE+Saline mice at p< 0.01 (**).
Figure 4.5. LDN inhibited general cellular proliferation (A,C), activation of astrocytes (B,D), and replication of activated astrocytes (E,F) in lumbar spinal cord from mice with established RR-EAE. Mice were evaluated at 5, 14, and 40 days of treatment. Ki67 stained (A) or GFAP stained (B) lumbar spinal cord from normal and RR-EAE mice treated for 14 days with LDN or saline. The numbers of positively stained proliferating cells (C) or activated astrocytes (D) over a 40-day period are shown. (E,F) Ki67 and GFAP double-labeled sections showing proliferating astrocytes within the central ventral white matter. The inset shows a proliferating astrocyte, demarcated by the red projections (TRITC = GFAP) surrounding a green nucleus (FITC = Ki-67). Insets in panels A and B are control specimens stained with secondary antibodies only; bars = 50 μm. The number of positively stained cells was recorded from at least 2 sections/mouse and 3-6 mice per group at each time point. Values represent means ± SEM. Significantly different from normal mice at p<0.01 (++) and p<0.001 (+++); significantly different from RR-EAE+Saline mice at p<0.01 (**), p<0.001 (***) and p<0.0001 (****).
Figure 4.6. Demyelination was reduced within the lumbar region of the spinal cord at 14 days. (A) Lumbar spinal cord sections were stained with Luxol fast blue and counterstained with neutral red. (B) Area of demyelination (percentage) for RR-EAE mice treated with LDN or saline on treatment days 14 and 40. Significantly different from normal values at $p<0.01$ (++) and from RR-EAE + Saline at $p<0.01$ (**).
References


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CHAPTER 5: MODULATION OF THE OGF-OGFR AXIS IN ESTABLISHED EAE AND EFFECTS ON CD4+ T LYMPHOCYTES
Rationale

T lymphocytes are known to play an important role in MS pathogenesis. OGF and LDN therapy reduce the number of infiltrating CD3\(^+\) lymphocytes present in RR-EAE spinal cord. Therefore, it is possible that OGF and LDN are targeting infiltrating immune cells peripherally as well as centrally by inhibiting proliferation of activated lymphocytes, working through the OGF-OGFr axis. Previous work has shown that the OGF-OGFr axis is present in splenic lymphocytes. However, little is known about the effects of this axis on the differential responses of cytokines associated with CD4\(^+\) T lymphocytes. The goal of the fourth aim was to determine whether OGF or LDN alter the Th effector responses of CD4\(^+\) T lymphocytes within the CNS in established EAE induced by PLP\(_{139-151}\).
Modulation of The OGF-OGFr Axis in Established Experimental Autoimmune Encephalomyelitis and Effects on CD4⁺ T Lymphocytes

Leslie A. Hammer, Hanspeter Waldner, Ian S. Zagon, and Patricia J. McLaughlin
Abstract

Multiple sclerosis is a debilitating autoimmune disease of the central nervous system, characterized by infiltrating myelin-reactive T lymphocytes and demyelinating lesions. Experimental autoimmune encephalomyelitis is the animal model widely utilized to study MS. EAE is mediated by CD4$^+$ T cells and can be induced in mice through immunization with a myelin antigen in EAE susceptible mice, such as PLP$_{139-151}$ in SJL/J mice. In this PLP induced EAE model in the SJL/J mouse, autoreactive CD4$^+$ T cells migrate from the periphery into the CNS where they are reactivated resulting in CNS damage (demyelination). Two CD4$^+$ Th effector cells known to have pathogenic roles in EAE and MS are Th1 and Th17 cells. These Th cells produce pro-inflammatory cytokines, IFN\(\gamma\), and IL-17, respectively. Anti-inflammatory Th2, IL-4 secreting cells, have been indicated to inhibit EAE exacerbation. However, given the inflammatory environment of EAE, Th2 effector cells are outnumbered by the Th1/Th17 cells. Regulatory CD4$^+$ T effector cells suppress immune reactions and have been demonstrated to be dysfunctional in MS patients. OGF, chemically termed met$^5$-enkephalin, is a negative growth factor that interacts with the OGFr. We can modulate the OGF-OGFr axis through exogenous administration of OGF or naltrexone, an opioid antagonist. Naltrexone, when given in small doses (low dose naltrexone) will block OGF from interacting with OGFr for a short period of time. We have previously demonstrated that modulation of the OGF-OGFr axis results in alleviation from relapse-remitting EAE and that CNS-infiltrating CD3$^+$ T cells are diminished with exogenous OGF or intermittent blockade with low dose naltrexone (LDN) administration. In this
paper, we aimed to determine whether OGF or LDN alter the Th effector responses of CD4\(^+\) T lymphocytes within the CNS in established PLP-EAE. We show here that the frequency (or numbers) of CD4\(^+\) T lymphocytes in the CNS of SJL/J mice with PLP induced EAE are decreased following treatment with OGF and not LDN at peak disease for 5 days. However, modulation of the OGF-OGFr axis did not result in changes to CD4\(^+\) Th effector cell responses in the CNS of EAE-diseased mice.
Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS), affecting approximately 2.3 million individuals worldwide. MS is characterized by infiltrating inflammatory cells, mainly autoreactive lymphocytes and macrophages into the CNS giving rise to an inflammatory cascade that results in demyelinated plaques. The etiology of MS remains elusive. Experimental autoimmune encephalomyelitis (EAE), the animal model of MS, is a CD4+ T cell mediated autoimmune disease that has pathophysiological similarities to MS, including infiltrating lymphocytes, demyelination, and neurodegeneration. EAE can be actively induced in the SJL/J mouse, a highly EAE-susceptible strain, through immunization with proteolipid peptide 139-151 (PLP139-151), resulting in activation of PLP-specific CD4+ T cells. The myelin-specific CD4+ T cells migrate to the CNS by crossing the blood brain barrier (BBB) and are reactivated resulting in a series of events leading to demyelination.

The effector cells and their associated cytokines that play a pathogenic and ameliorative role in EAE are Th1 (IFNγ), Th17 (IL-17) and Th2 (IL-4), and regulatory T cells (Foxp3), respectively. Proinflammatory cells, Th1 and Th17, are found in high frequency in lymph nodes, spleens and spinal cords of EAE mice during acute disease. Prior to the discovery of Th17 cells, secreting IL-17, IFNγ secreting Th1 cells were considered the primary cell responsible for the development of EAE.

In 2005, Langrish et al., examined the role of IL-23 in EAE by inducing disease in IL-23p19 deficient mice. The IL-23p19−/− mice were resistant to EAE, suggesting an important role for IL-23 in autoimmune diseases. Furthermore, CD4+ T cells isolated
from IL-23 deficient mice displayed reduced IL-6, TNF, and undetectable IL-17 production, despite the presence of IFNγ within the CNS. In vitro studies confirmed these findings, demonstrating that in vivo antigen primed lymphocytes cultured in the presence of IL-23 resulted in the production of IL-17 from CD4+ Th17 cells. These findings suggested that Th1 is not a requirement for EAE development, and that Th17 is responsible for EAE development. However, more recent studies indicate that both Th1 and Th17 are involved in disease manifestation.

A primary focus of MS therapeutic research has been to shift from the T1/Th17 to a Th2/regulatory T cell (Treg) paradigm. Th2 and Treg CD4+ T cells are anti-inflammatory cells that help suppress immune responses, a response that is impaired in autoimmune disease such as EAE/MS. Animal studies have confirmed that IL-4, the cytokine secreted by Th2 cells, alleviates EAE development. Constantinescu and colleagues compared MBP-stimulated lymphocytes from EAE-susceptible SJL/J mice with EAE-resistant BALB/c mice, looking specifically at Th1 and Th2 cytokines, IFNγ and IL-4 production. They found that MBP-stimulated SJL/J lymphocytes produced predominantly IFNγ and insignificant amounts of IL-4, while BALB/c mice lymphocytes exhibited the inverse. This supports IL-4 as an important cytokine for immune protection.

Tregs, which express Foxp3, have been studied thoroughly throughout the years in EAE models proving their ability to prevent disease development and lessening disease severity. Furthermore, Tregs have been identified in MS patients' peripheral
blood and were found to have diminished suppressive functions, indicating an inhibited ability to control an inflammatory event.\textsuperscript{15}

Opioid growth factor (OGF), chemically termed met\textsuperscript{5}-enkephalin, is a tonically active neuropeptide, that is produced in an autocrine fashion and secreted.\textsuperscript{20} OGF has a potent inhibitory growth effect mediated through interaction with the opioid growth factor receptor (OGFr).\textsuperscript{20} OGFr is a non-classical opioid receptor that is ubiquitously expressed.\textsuperscript{20} It has previously been demonstrated that the OGF-OGFr axis is present in T and B lymphocytes \textit{in vitro}.\textsuperscript{21,22} Stimulated T and B cells were treated with OGF and displayed a significant decrease in proliferation, indicating that the OGF-OGFr axis is present in activated T and B cells and can be modulated to impair proliferation.\textsuperscript{21,22} We have previously demonstrated that modulation of the OGF-OGFr axis with both OGF and low doses of naltrexone (LDN) decreases clinical disease severity, as well as diminishes the pathological damage found within the spinal cord of both chronic and relapse-remitting (RR) EAE mice.\textsuperscript{23-27} Since it is known that CD4\textsuperscript{+} Th cells play a dominant role in the development and/or alleviation of EAE pathogenesis, and that the OGF-OGFr axis is present in T cells, we sought to determine whether modulation of this axis alters CD4\textsuperscript{+} T lymphocyte effector responses. The aim of this study is to determine whether modulation of the OGF-OGFr axis with OGF or LDN administration alters the immune response centrally (treatment from disease onset). We treated mice with established PLP-induced EAE with OGF or LDN for five days to determine whether modulation of the OGF-OGFr axis alters Th effector responses within the CNS. Brains and spinal cords were harvested at peak disease and intracellular cytokine and
transcription factor staining of CD4+ T lymphocytes (IFNγ, IL-4, IL-17 and Foxp3) was carried out and cells were analyzed by flow cytometry.
Materials and Methods

Animals

6-8 week old Female SJL/JOrllcoCrl mice (Charles River Labs, Wilmington, MA) were housed 5 per cage under standard conditions in a separate room from other rodents and acclimated for one week prior to disease induction; food and sterile water were available ad libitum. As the course of EAE progressed, soft food and HydroGel (ClearH₂O, Portland, ME) were placed on the floor of the cages. All experiments were conducted in accordance with the National Institute of Health guidelines on animal care, and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Induction of EAE

Experimental autoimmune encephalomyelitis (EAE) was induced in 6-8 week SJL/JOrllcoCrl mice via subcutaneous immunization with 100 µg of myelin proteolipid protein 139-151 (PLP₁₃₉₋₁₅₁) (Peptides International, Louisville, KY), as previously published.²⁶,²⁷ Mice were injected with an emulsion containing equal parts PLP₁₃₉₋₁₅₁ in 0.15 ml sterile phosphate buffered saline (PBS) and 0.15 ml incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO) supplemented with 250 µg mycobacterium tuberculosis (H37RA, Difco Laboratories, Detroit, MI). Mice were lightly anesthetized with 3% isoflurane (Vedco, Inc., St. Joseph, MO) and injected subcutaneously (s.c.) at three sites on the back with 100 µl (total volume of 300 µl) of PLP₁₃₉₋₁₅₁. Intraperitoneal (i.p.) injections of 200 ng pertussis toxin (List Biological Laboratories, INC., Campbell, CA) were administered on days 0 and 2 post immunization.
Drug Treatments

Mice were randomly assigned to treatment groups beginning at time of established EAE. Animals received i.p. injections (0.1 ml) of 10 mg/kg OGF (Polypeptide Laboratories, Torrance, CA) (EAE + OGF), 0.1 mg/kg naltrexone (EAE + LDN) (Sigma-Aldrich, Indianapolis, IN) or sterile saline (EAE + Saline).

Behavioral Observations

Mice were observed daily for clinical disease presentation, by two individuals (one observer masked to treatment, scores were reconciled at time of observation). Behavior was assessed according to previously published protocols, utilizing a 10-point scale, which consists of analysis of gait, limb strength, tail tonicity and righting reflex.\textsuperscript{24,26,27} Disease onset was defined as the second consecutive day that a mouse had a behavioral score of 0.5 or greater.

Isolation of Mononuclear Cells from CNS

Mice were anesthetized with a cocktail of ketamine (30 mg/kg), xylazine (5 mg/kg) and acepromazine (2 mg/kg) diluted in sterile water and perfused through the left ventricle with cold PBS. Brains and spinal cords were collected and mononuclear cells were isolated following published protocols.\textsuperscript{28,29} CNS tissue was digested in DMEM supplemented with 2.5 mg/ml Collagenase D (Roche Diagnostics, Indianapolis, IN) and 10 μg DNAseI (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. The digestion was deactivated with 0.5 mM EDTA (Affymetrix USB, Cleveland, OH). A single cell suspension was prepared utilizing a 70 μm cell strainer, and centrifuged for 5 min at 1500 rpm at room temperature (RT), fresh percoll gradients were prepared. Cells were
resuspended in 4 ml of 70 percent percoll and gently overlayed with 4 ml of 37 percent percoll (plus 0.5 ml DMEM for color contrast) and then 4 ml of 30 percent percoll and centrifuged at 1800 rpm (500g), for 30 min at RT (slow accelerator & break off). The interphase between 70 and 37% layers including the distinct white ring in the middle was collected, leaving the RBCs at the bottom of the tube behind, and transferred to a new 15 ml conical tube and washed thoroughly with 0.5mM PBS/EDTA. Cells were resuspended and counted using a hemacytometer (1:5 dilution in trypan blue).

**Intracellular Cytokine Staining**

Cells (1-2 x 10^6) were stimulated for 4 hours at 37°C in a 6-well plate with 3 ml IMDM, 5ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 500ng/ml ionomycin (Sigma-Aldrich) in the presence of 2ul/well brefeldin A (eBioscience, San Diego, CA). Harvested cells were spun down and resuspended in 2.4g2 Fc block and placed on ice for 15 min. Cells were surface stained with anti-CD4 FITC (1:100, RM4-5, eBioscience) for 15 min. After surface staining, cells were fixed and permeabilized (Fix & Perm kit, GAS003, Invitrogen, Camarillo, CA; or Foxp3 Stain kit, eBioscience) and stained with the following antibodies: 1:100, Foxp3 APC (FJK-16s), IL-4 PE (11B11), IFNγ APC (XMG1.2), and IL-17A PerCP Cy 5.5 (eBio17B7), and appropriate isotype controls (all antibodies were from eBioscience). Cells were analyzed on the FacsCalibur (BD biosciences, San Jose, CA) and data was analyzed utilizing BD CellQuest Pro software (BD bioscience).
Immunohistochemistry

EAE mice were sacrificed after 40 days of treatment and perfused with 4% paraformaldehyde (PFA) and lumbar spinal cords were collected and embedded in OCT compound and stored at -80°C, according to previously published protocols.26,27 Spinal cords were sectioned at 10 μm on a cryostat and collected on glycerol-subbed slides. Slides were brought up to RT and washed twice in SPB (sodium phosphate buffer) and blocked for 2 hrs with 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in SPB at RT. Spinal cords were double labeled with primary antibodies rabbit anti-CD3 and rat anti-CD4 (1:200, Abcam, Cambridge, MA) at 4°C overnight (at least 20 hrs). Slides were washed in 1% NGS/SPB twice for 15 min each, then blocked for 1 hr at RT with 5% NGS/1% BSA in SPB. Slides were incubated for 1 hr at 4°C with secondary goat-anti-rabbit TRITC (1:1000) and goat-anti-rat FITC (1:1000, Molecular Probes, Grand Island, NY) antibodies and DAPI (1:5000). Slide Book Pro was used to acquire images at 20x magnification of the central ventral white matter. ImageJ (NIH) software was utilized for analysis. Positive cells were identified as a DAPI labeled nuclei surrounded by both TRITC and FITC. Cells were counted per field and recorded.

Statistical Analysis

Data was analyzed using one-way analysis of variance (1-way ANOVA) or student’s T test where appropriate in GraphPad Prism 6 software (La Jolla, CA). A p-value less than 0.05 was considered significant.
Results

Both OGF and LDN are capable of diminishing clinical disease scores, as well as decreasing the number of CD3$^{+}$ T lymphocytes within the lumbar spinal cord in an established EAE model.\textsuperscript{26,27} These studies were limited to IHC analysis of the central ventral white matter of the lumbar spinal cord, which gave us insight into how OGF and LDN alter EAE. However, we were interested in exploring if these findings were consistent throughout the CNS of EAE mice, or limited to the lumbar spinal cord, and whether OGF or LDN were capable of altering the CD4$^{+}$ effector T lymphocytes during the effector phase of disease. Therefore, we decided to treat mice after they developed clinical disease (established disease) and examine the CNS at peak disease where the highest numbers of CNS infiltrates should be detected.

Behavior and General Observations

No animals died from immunizations; one mouse developed a small lesion at an injection site. Five days of treatment from time of disease onset had no effect on clinical presentation or behavioral development (Figure 5.1A), consistent with previous studies.\textsuperscript{26,27}

Modulation of the OGF-OGFr Axis in Established EAE has No Effect on the Number of CNS-Infiltrating Mononuclear Cells

Five days of OGF (10 mg/kg) or LDN (0.1 mg/kg naltrexone) treatment had no effect on the number of mononuclear cells in the CNS of mice with established EAE. Total cell numbers were calculated per tube, each tube contained the brain and spinal cord from two mice. There were $2.7 \times 10^6 \pm 5.6 \times 10^5$ (mean $\pm$ SEM) mononuclear cells
per CNS tissue for EAE + Saline mice, while $2.1 \times 10^6 \pm 1.7 \times 10^5$ mononuclear cells per EAE mouse treated with OGF and $2.5 \times 10^6 \pm 2.2 \times 10^5$ mononuclear cells per mouse were collected from EAE + LDN mice (Figure 5.1B).

However, the number of infiltrated mononuclear cells into the CNS of EAE mice correlated to clinical disease scores (Figure 5.1C), determined by utilization of the Spearman correlation analysis (coefficient = 0.5925 and a p-value of 0.0096). All EAE mice were combined for behavioral correlation analysis.

**OGF Reduces the Number of CD4$^+$ T Lymphocytes in the CNS**

Modulation of the OGF-OGFr axis for 5 days of treatment with LDN after clinical disease developed resulted in increased numbers of CD4$^+$ T lymphocytes in the brain and spinal cord, whereas the numbers of CD4$^+$ T lymphocytes were significantly decreased in EAE mice that received OGF compared to saline treated controls. EAE + Saline mice had an average of $5.2 \times 10^5$ CD4$^+$ T lymphocytes, EAE + LDN mice had an average of $7.1 \times 10^5$ and EAE + OGF mice had an average of $3.3 \times 10^5$ CD4$^+$ T lymphocyte infiltrates (Figure 5.2B). The average percentage of cells from EAE + Saline mice that were CD4 positive was 19.8%, compared to EAE + OGF at 13.2% and EAE + LDN at 27.8% (Figure 5.2A).

**Modulation of the OGF-OGFr Axis Does Not Play a Role in CD4$^+$ T Lymphocyte Effector Responses**

Intermittent blockade of the OGF-OGFr axis or exogenous OGF administration for 5 days in established EAE did not alter the presence of CD4$^+$ Th1, Th2 (data not shown), Th17 nor Treg infiltrates within the CNS (Figure 5.3B).
The Numbers of CD3⁺CD4⁺ T Lymphocyte Infiltrates in the Lumbar Spinal Cord are Limited by Both OGF and LDN

Lumbar spinal cord sections were stained with antibodies against CD3 and CD4 after 40 days of treatment with OGF, LDN or saline to determine the presence of CD4⁺ T lymphocytes. The number of CD4 positive T cells within the central ventral white matter (CVWM) of the lumbar spinal cord were decreased after 40 days of treatment with OGF in EAE mice (17.3 ± 5.7 CD3⁺CD4⁺ cells, mean ± SEM) and LDN (10.3 ± 2.5 CD3⁺CD4⁺ cells) treated mice compared to Saline controls (84.9 ± 19.8 CD3⁺CD4⁺ cells) (Figure 5.4B).
Discussion

In an established relapse-remitting EAE model, we have previously shown that OGF and LDN decreased the presence of CD3 positive infiltrates in lumbar spinal cord sections after 40 days of treatment, but have no effect after 5 days of treatment compared to saline treated controls. In this study we sought to determine whether OGF or LDN had any differential effects on CD4+ T lymphocyte cytokine responses, given that these cells predominate CNS infiltrates in both the acute and relapse phases of EAE in SJL/J mice.

We have demonstrated that mice with established EAE that were treated with OGF for 5d from the day of disease onset exhibit diminished numbers of mononuclear cells in their brain and spinal cord. This correlated well with flow analysis showing that the absolute number of CD4+ T cells were significantly reduced in EAE + OGF mice compared to saline and LDN treated EAE mice. Immunohistochemical evaluation of lumbar spinal cord sections from OGF and LDN treated mice with established EAE, revealed decreased numbers of CD3+CD4+ T lymphocytes in the central ventral white matter after 40 days of treatment compared to saline controls. Even though OGF decreased the number of CD4+ T cell infiltrates, it does not alter the effector CD4+ T cell responses. The frequency of IL-4+CD4+ T cells were undetectable, which is expected due to the inflamed environment.

Our laboratory has previously shown that OGF administration in vitro to PHA-stimulated splenic T lymphocytes decreased proliferative activity. However, OGF did not have an effect on non-stimulated cell cultures. We also demonstrated that
naltrexone had no effect on either PHA-stimulated or non-stimulated T lymphocytes, while in other cell types naltrexone increases proliferation.\textsuperscript{21} These data suggest that the OGF-OGFr axis is only active once T cells become activated. In this study, we examined the actions of the OGF-OGFr axis on CD4\textsuperscript{+} T lymphocytes during the effector phase of EAE by waiting until disease onset to administer treatments. The results of this approach were decreased CD4\textsuperscript{+} T lymphocytes in OGF-treated EAE mice and increased CD4\textsuperscript{+} T lymphocytes in EAE mice treated with LDN. No treatment altered the Th effector cell responses in EAE after only 5 days of treatment. If the OGF-OGFr axis is solely functioning as a mediator of proliferation, then modulation of the axis should not alter T cell subsets individually. Which is what we are observing here in this SJL/J-PLP\textsubscript{139-151} model of EAE.

In order to further establish the actions of the OGF-OGFr axis in PLP-reactive CD4\textsuperscript{+} T cells, future studies should utilize other strategies, including adoptive transfer of CFSE-labeled PLP-specific CD4\textsuperscript{+} T cells or CD4\textsuperscript{+} T cells from PLP TCR transgenic mice.\textsuperscript{31} This would allow us to examine whether modulation of the OGF-OGFr axis affects the trafficking of myelin-specific lymphocytes to the CNS. To allow investigating the effects of OGF without the possible interactions of OGF with the classical opioid receptors, it would be necessary to utilize a triple opioid receptor (MOR, DOR and KOR) knock-out animal model of EAE.
Figure 5.1. Modulation of the OGF-OGFr axis in established EAE. SJL/J mice were immunized with PLP\textsubscript{139-151} and allowed to develop disease. Mice were treated on the second consecutive day of clinical disease with 10 mg/kg OGF (EAE + OGF), 0.1 mg/kg naltrexone (EAE + LDN) or saline (EAE + SALINE). (A) Mean clinical disease scores over the course of the 14-day experiment. (B) Total number of mononuclear cells isolated from CNS (brain + spinal cord) are shown. (C) Correlation between clinical disease score and total mononuclear cells of all EAE mice is shown, Spearman r = 0.5925, p = 0.0096 (**).
Figure 5.2. Modulation of the OGF-OGFr axis alters the numbers of CD4$^+$ T lymphocytes present in the CNS of mice with established EAE. (A) Percent of total cells that are CD4$^+$ isolated from CNS tissue of EAE mice treated with OGF, LDN or saline for 5 days from disease onset. (B) Number of CD4$^+$ T lymphocytes isolated from the CNS of EAE mice. Significantly different from EAE + SALINE at $p < 0.01$ (**), $p < 0.001$ (***)$,$ and $p < 0.0001$ (****). Significantly different from EAE + OGF at $p < 0.0001$ (++++).
**Figure 5.3.** Effector CD4+ T lymphocytes isolated from the brain and spinal cord of EAE mice after 5 days of treatment (10 mg/kg OGF, 0.1 mg/kg naltrexone (LDN), or saline), corresponding to 15 days post immunization. (A) Intracellular cytokine staining of IFNγ, IL-17, and transcription factor staining of Foxp3 in mononuclear cells isolated from CNS tissue gated on live cells after *ex vivo* stimulation with PMA/ionomycin and brefeldin A for 4h. (B) Percentage of CD4+ T lymphocytes that are positive for IFNγ, IL-17, and Foxp3 are shown.
**Figure 5.4.** CD3⁺CD4⁺ T lymphocytes in the lumbar spinal cord of established EAE mice treated for 40 days with OGF, LDN or saline. (A) Lumbar spinal cord were stained with antibodies for CD3 (TRITC) and CD4 (FITC) after 40 days of treatment, 20x (left) and 40x (right) magnification; CD3⁺/CD4⁺ cell (arrow). Inset is stained with secondary antibody only as a control. (B) Number of CD3⁺/CD4⁺ cells in the CVWM of lumbar spinal cord after 40 days of treatment. Significantly different from EAE + SALINE at p < 0.01 (**).
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Fulminant spontaneous autoimmunity of the central nervous system in mice transgenic
for the myelin proteolipid protein-specific T cell receptor. *PNAS* 97(7):
CHAPTER 6: DISCUSSION
6. Discussion

6.1 Summary

Previous work in our laboratory examined the role of the OGF-OGFr axis in a chronic EAE model of MS, through treatments with exogenous OGF, intermittent blockade with LDN, and complete blockade with high dose naltrexone (HDN). The outcome of these studies was that OGF and LDN altered the disease course of mice with EAE when treated from the time of disease induction. However, only OGF treatment significantly ameliorated disease in MOG-induced EAE when treatment began after established disease. HDN, on the other hand, did not alter behavioral profiles or spinal cord morphology of chronic EAE regardless of whether it was administered at the time of induction or established disease. These results are encouraging for OGF as a possible treatment option for primary progressive MS. However, only approximately 10 percent of individuals with MS develop the primary progressive form of disease, while 85 percent of patients experience the relapse-remitting disease course. Therefore, modulation of the OGF-OGFr axis needed to be assessed in a relapse-remitting form of EAE and led to the central hypothesis of this dissertation. The studies presented here support the OGF-OGFr axis as an important therapeutic target for the treatment of autoimmune diseases such as MS. Specific aim one established a working model of relapse-remitting EAE in the SJL/J mouse immunized with PLP139-151. Mice were treated with OGF or saline from the time of immunization, and data demonstrated that OGF-treated mice experienced more time in remission and had decreased spinal cord histopathology. Specific aims 2 and 3 were focused on modulation of the OGF-OGFr
axis with exogenous OGF or LDN in mice with established RR-EAE. Both treatment regimens altered clinical disease manifestations. Both exogenous OGF administration, as well as, intermittent blockade of the OGF-OGFr axis impacted the severity of spinal cord histopathology, resulting in reductions in demyelination, T cell infiltration, activated astrocytes, microglia/macrophages, and proliferation (Table 6.2). In addition, the utilization of flow cytometry provided evidence that exogenous OGF, administered for only 5 days, can reduce the number of CD4+ T lymphocytes within the CNS of mice with established EAE compared to saline-treated and LDN-treated EAE mice.

These pre-clinical studies add to the body of evidence that modulation of the OGF-OGFr axis not only ameliorates chronic EAE, but also relapse-remitting EAE, and could be an effective therapeutic option for all MS patients. These data warrant further clinical studies to determine the efficacy of both OGF and LDN in the treatment of MS.

6.2 Disease Model

6.2.1 Chronic Versus Relapse-Remitting EAE

The studies in this dissertation were designed to generate the EAE model in SJL/J mice that present with the relapsing-remitting form of EAE; similarities in this animal model resemble the clinical disease manifestations with RR-MS. Previous studies in our laboratory examined the effects of modulating the OGF-OGFr axis in a chronic EAE model with positive results. The chronic model uses the C57BL/6 mouse and involves immunization with MOG_{35-55}, characterized by ascending paralysis that peaks around 18 days post induction and then plateaus. The relapsing-remitting model utilizes the SJL/J mouse immunized with PLP_{139-151} and is characterized by ascending
paralysis followed by varying stages of recovery. Both animal models present with pathologies similar to those associated with MS, including demyelination, T cell infiltration of the blood brain barrier, activated astrocytes and microglia, as well as, axonal and neuronal damage.

6.2.2 Limitations of the SJL/J Disease Model

In 2010, Summers deLuca and colleagues examined PLP$_{139-151}$ EAE in SJL/J mice from four vendors (Harlan Laboratories, The Jackson Laboratory, Charles River Laboratories and Taconic Farms) to address the inconsistencies reported in the SJL/J + EAE animal model. They found that the vendor represented the most significant degree of variability. Of particular interest to our studies, the Charles River SJL/J mouse had a bipartite response to PLP$_{139-151}$. Approximately 30% of the Charles River mice had a chronic disease course and an absence of remissions. The differences noted in the Charles River mice may play a role in the bipartite response reported in our established experiments. We have noted that 40-50% of RR-EAE mice receiving OGF or LDN have a more severe disease course that closely resembles that of saline treated controls. Environment did not appear to play a role. All mice were housed with at least one other mouse and no cage contained all responders or all non-responders. Any mouse with a severe tail wound was omitted from the study. The order in which mice received injections (initial immunization or treatments) did not affect responses. Whether the varied responses Charles River mice had to PLP$_{139-151}$ noted by Summers de Luca$^6$ directly relate with OGF and LDN treatments is unknown. Future studies should consider using a different vendor for SJL/J mice such as The Jackson
Laboratories. Aside from the possible limitations with the choice of vendor, the induction protocol utilized in these studies has drawbacks. Throughout this dissertation EAE was induced through immunization with PLP\textsubscript{139-151} in CFA. It is possible that small variations during injections could have altered disease onset and severity of disease, thus affecting the outcomes of treatment with LDN or OGF. The lot of the peptide used for immunization is also important for development of EAE. Indeed we found this to be the case in one experimental run where nearly all mice remained free of disease despite routine immunization procedures.

Passive induction of EAE through adoptive transfer of encephalitogenic CD4\textsuperscript{+} T cells into naïve mice potentially would result in fewer variations and would allow for wider range of opportunities to explore the mechanism behind the OGF-OGFr axis in EAE. This model would allow for the opportunity to closely examine the possible migration and proliferation properties of OGF or LDN.

Despite limitations with animal models of MS, EAE, though imperfect, is the best-known model to study MS in order to gain further insights into disease mechanisms and identifying new therapies.

6.2.3 Induction Versus Established Disease

The first aim of this dissertation addressed the effects of OGF treatment from the time of disease induction. Treating from time of disease induction allowed us to fully examine how modulation of the OGF-OGFr axis could impact disease outcome from the pre-effector stage of disease. While this proved effective, it does not correlate well with clinical disease. Therefore, we chose to wait until the effector phase of disease, when
mice displayed clinical signs of disease through behavioral presentation. Before animals could be placed into a treatment group they had to have a behavioral score greater than or equal to 0.5 for two consecutive days. This most closely represents clinically isolated syndrome (CIS), where patients present with initial symptoms that could potentially develop into MS. Treating mice from time of established disease, therefore gives us a better understanding of how OGF would be an effective therapeutic option for CIS patients or those patients diagnosed early in the disease course of MS.

Perhaps a better examination of the modulation of the OGF-OGFr axis for translational purposes would be to delay treatment until the mice reach peak disease, around 14 to 15 days post immunization; potentially waiting until the mouse exhibits hindlimb paralysis in at least one limb. This would limit the chance that an initial disease score would affect the outcome of treatment effectiveness.

**6.2.4 Behavior Scoring**

The standard behavior scoring system utilized in EAE studies is a 5-point scale. However, our laboratory utilizes a 10-point scale in order to obtain more detailed behavioral analysis [Table 6.1]. The 10-point scale expands on the 5-point scale by incorporating scoring of each individual limb, degree of righting reflex, gait abnormalities as well as degree of tail tone loss. This is an advantage when examining a potential drug’s effectiveness on behavioral outcomes, since it allows for a more accurate EAE score.
<table>
<thead>
<tr>
<th>EAE Score</th>
<th>Clinical Sign</th>
<th>EAE Clinical Parameters</th>
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<tr>
<td></td>
<td></td>
<td><strong>EAE Score</strong></td>
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<tr>
<td>0</td>
<td>Normal mouse</td>
<td>Normal mouse, no signs of disease</td>
</tr>
<tr>
<td>1</td>
<td>Limp tail or hind limb weakness</td>
<td>Tail tonicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drags, has tone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No tone</td>
</tr>
<tr>
<td>2</td>
<td>Limp tail and hind limb weakness</td>
<td>Gait abnormalities</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal Gait</td>
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<tr>
<td></td>
<td></td>
<td>Wobbly (Slight)</td>
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<tr>
<td></td>
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<td>Moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe</td>
</tr>
<tr>
<td>3</td>
<td>Partial hind limb paralysis</td>
<td>Limb strength</td>
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<tr>
<td></td>
<td></td>
<td>(Each limb scored individually)</td>
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<tr>
<td></td>
<td></td>
<td>Fault</td>
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<td></td>
<td></td>
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<td>4</td>
<td>Complete hind limb paralysis</td>
<td>Righting reflex</td>
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<td></td>
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<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>5</td>
<td>Moribound or Death from EAE</td>
<td>Death from EAE</td>
</tr>
</tbody>
</table>

Table 6.1. Differences in 5-point and 10-point EAE clinical disease scoring systems. 5-point scale modified from Miller et al., 2007.³

6.3 Exogenous OGF versus Intermittent Blockade of OGFr in RR-EAE

Similar to previous studies in the chronic EAE model, exogenous OGF had a greater impact of alleviating PLP-induced EAE compared to LDN treatment [Table 6.2]. The differences observed in EAE mice treated with exogenous OGF or intermittent blockade of the OGF-OGFr axis through LDN, may be due to the level of activity of the axis in the naïve mouse or LDN’s non-specific blockade of opioid receptors. Further research is required to elucidate the mechanism behind the observed differences of OGF and LDN treatment in EAE.
<table>
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<tr>
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<th>PLP + OGF</th>
<th>RR-EAE + OGF</th>
<th>RR-EAE + LDN</th>
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</thead>
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<td><strong>Behavior</strong></td>
<td>![Down Arrow]</td>
<td>![Down Arrow]</td>
<td>![Down Arrow]</td>
</tr>
<tr>
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<td>![Green Arrow]</td>
<td>![Green Arrow]</td>
<td>![Green Arrow]</td>
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<tr>
<td>T-Lymphocytes</td>
<td>![Down Arrow]</td>
<td>![Down Arrow]</td>
<td>![Down Arrow]</td>
</tr>
<tr>
<td>Activated Astrocytes</td>
<td>![Green Arrow]</td>
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<td>![Green Arrow]</td>
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<tr>
<td>Demyelination</td>
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<td>Proliferation</td>
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<td>Astrocyte proliferation</td>
<td>![Green Arrow]</td>
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**Table 6.2.** Comparison of effects of OGF and LDN on disease manifestations of mice with RR-EAE. Arrows indicate the level of significance, wider arrows equal greater significance.

### 6.4 Modulation of the OGF-OGFr axis effects on neuropathology

Exogenous OGF treatment in RR-EAE from time of disease induction began to have positive effects on neuropathological aspects of disease after fourteen days of treatment, which corresponds with peak disease. However, cell proliferation as identified with Ki67 staining was not inhibited until 55 days of treatment. In comparison, 5 days of OGF treatment of mice with RR-EAE from time of disease onset, which corresponds with peak disease, resulted in reductions in activated astrocytes and had no effects on other neuropathological aspects of disease at that time point. After 14 days of treatment, corresponding to the first remission, OGF decreased the level of proliferating cells as labeled by Ki67 treatment. This time point was not examined in the
first study where treatments were given at the time of immunization. It would however, be interesting to know if these effects would be greater at the same time point in the induction model, correlating to day 24 post immunization. Interestingly, lymphocytes and microglia/macrophages were not impacted until mice were treated for 40 days from disease onset. These results correlate well with demyelination, in that mice treated with OGF in established RR-EAE after 40 days had reduced demyelination.

Our data from studies on OGF treatment of mice with RR-EAE show that exogenous OGF therapy requires more than 5 days of consecutive treatments before positive effects can be seen. Histopathological analyses of lumbar spinal cord sections reveal that modulation of the OGF-OGFr axis has the greatest effect on the number of activated astrocytes compared to numbers of microglia/macrophages and infiltrating T lymphocytes. This observation aligns nicely with previous studies in vitro on astrocytes and lymphocytes investigating the role and presence of the OGF-OGFr axis. Our laboratory demonstrated that the OGF-OGFr axis is present and active in astrocytes. We have also demonstrated that in activated lymphocytes only, exogenous OGF was capable of inhibiting proliferation, while naltrexone had no effects. In the present study, exogenous OGF administration in established RR-EAE reduced the levels of activated astrocytes after only 5 days of treatment while only affecting T lymphocytes after 40 days of treatment. This speaks somewhat to the presence and activity of the axis in naïve cells versus activated cells. Suggesting that the more active OGF-OGFr axis a particular cell has, the more effective exogenous OGF treatment will be. Therefore, it
would be important to examine the OGF-OGFr axis in microglia and macrophages, due to their important role in disease manifestations.

It is valuable to also compare the effects of intermittent blockade of the OGF-OGFr axis and its effects on neuropathology to exogenous OGF treatment in established RR-EAE. LDN began to have positive effects after 5 days of treatment on Iba-1 and GFAP reactivity in lumbar spinal cord sections of RR-EAE mice. Interestingly, LDN did not continue to have therapeutic effects on microglia after 14 and 40 days of treatment, while F4/80^+Iba-1^+ cells were reduced at both 14 and 40 days of treatment. This may indicate varied activity in endogenous opioid-opioid receptor interactions in microglia and macrophages. LDN significantly reduced demyelination of lumbar spinal cord sections after 14 days of treatment, corresponding to first remission. However, it had no effect after 40 days of treatment, which may be due to the bipartite behavioral response observed in LDN-treated mice. While LDN responders display significant reductions in demyelination compared to the LDN non-responders, neither group was significantly different from saline-treated mice. Suggesting that the behavioral responses to LDN treatment may not be solely responsible for the lack of protection against demyelination, after 40 days of treatment.

**6.5 Future Directions**

The results of this body of research further support the use of OGF and LDN as potential therapeutic options for MS patients. However, due to limitations with our choice of animal model, including strain of mouse and method of disease induction, future studies should include the evaluation of the OGF-OGFr axis in better suited
animal models for analysis of mechanistic activities in EAE. In the current SJL/J EAE model, where we utilized active immunization with PLP_{139-151}, OGF has the potential of interacting with the classical opioid receptors and some of the effects we have observed could potentially be due to those interactions. In order to clarify that the effects reported here are due to modulation of the OGF-OGFr axis future studies should utilize a triple-opioid receptor knock-out mouse model, where the classical opioid receptors are not present. This model would isolate the OGF-OGFr axis and reveal pathways that may have been masked by the wild-type SJL/J and C57BL/6 mouse models of EAE.

This dissertation has demonstrated that exogenous OGF administered in EAE for 5 days after clinical disease decreases the amount of CD4^{+} T lymphocytes within the CNS. Whether this is a result of migration or proliferation of T cells remains unclear, however, studies utilizing an adoptive transfer model where encephalitogenic CD4^{+} T cells labeled with CFSE are injected into a naïve host to develop EAE may provide answers to these questions. This would allow for the encephalitogenic T cells to be followed while modulating the OGF-OGFr axis with exogenous OGF or intermittent blockade of OGFr with LDN. A more thorough study should be conducted to evaluate the effects on CNS-infiltrating CD4^{+} T lymphocytes at other time points such as first remission and first relapse.

One area of EAE pathology that has yet to be studied in our laboratory is examining the OGF-OGFr axis in macrophages. We have yet to characterize the presence of the axis in macrophages. Questions that need to be answered include: (i) is the OGF-OGFr axis present and tonically active in macrophages, (ii) does OGF inhibit
their proliferation, and (iii) does modulation of the OGF-OGFr axis alter the degree of macrophage infiltration into the CNS?

These questions should be addressed in cell culture as well as in an EAE animal model. Understanding the impact of exogenous OGF or intermittent blockade with LDN on macrophages will further our knowledge of how modulation of the OGF-OGFr axis alters EAE disease course.

It would be interesting to study the effects of OGF and LDN treatment on lesion formation in EAE utilizing MRI throughout the length of the experiment in order to truly follow an animal's disease course without sacrificing the animal for histopathology at early time points. Furthermore, some MS patients are currently taking LDN, although it has not been FDA approved as an MS therapy. Investigations should be completed to further elucidate how LDN is functioning in MS, such as, are these patients experiencing decreased lesion formation or just feeling more energetic due to upregulation of endorphins and dynorphins? This could potentially be accomplished by measuring endorphin levels in the blood before and after taking LDN as well as longitudinal studies with MS patients to identify changes in lesions or the development of new lesions. Therefore, a clinical trial would be most beneficial.
6.6 References


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