Research Report

Prevention and diminished expression of experimental autoimmune encephalomyelitis by low dose naltrexone (LDN) or opioid growth factor (OGF) for an extended period: Therapeutic implications for multiple sclerosis

Kristen A. Rahn, Patricia J. McLaughlin, Ian S. Zagon⁎

Department of Neural and Behavioral Sciences, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA

ARTICLE INFO

Article history:
Accepted 12 January 2011
Available online 20 January 2011

Keywords:
Experimental autoimmune encephalomyelitis
Multiple sclerosis
Opioid
Opioid growth factor
Opioid growth factor receptor
Autoimmune diseases

ABSTRACT

Endogenous opioids inhibit the onset and progression of experimental autoimmune encephalomyelitis (EAE) with 30 days of treatment. This study examined the long term effects of the opioid growth factor (OGF, [Met5]-enkephalin) and a low dose of the opioid antagonist naltrexone (LDN) on expression of myelin oligodendrocyte glycoprotein (MOG)-induced EAE. C57BL/6 mice began receiving daily injections of 10 mg/kg OGF (MOG+OGF), 0.1 mg/kg naltrexone (MOG+LDN), or saline (MOG+Vehicle) at the time of EAE induction and continuing for 60 days. In contrast to 100% of the MOG+Vehicle group with behavioral symptoms of EAE, 63% and 68% of the MOG+OGF and MOG+LDN mice expressed disease. Both severity and disease indices of EAE in OGF- and LDN-treated mice were notably decreased from MOG+Vehicle cohorts. By day 60, 6- and 3-fold more animals in the MOG+OGF and MOG+LDN groups, respectively, had a remission compared to MOG+Vehicle mice. Neuropathological studies revealed i) astrocyte activation and neuronal damage as early as day 10 (prior to behavioral symptoms) in all MOG-injected groups, ii) a significant reduction of activated astrocytes in MOG+OGF and MOG+LDN groups, respectively, had a remission compared to MOG+Vehicle mice. Both severity and disease indices of EAE in OGF- and LDN-treated mice were notably decreased from MOG+Vehicle cohorts. By day 60, 6- and 3-fold more animals in the MOG+OGF and MOG+LDN groups, respectively, had a remission compared to MOG+Vehicle mice. Neuropathological studies revealed i) astrocyte activation and neuronal damage as early as day 10 (prior to behavioral symptoms) in all MOG-injected groups, ii) a significant reduction of activated astrocytes in MOG+OGF and MOG+LDN groups, respectively, had a remission compared to MOG+Vehicle mice at day 30, and iii) no demyelination on day 60 in mice treated with OGF or LDN and not displaying disease symptoms. These results indicate that treatment with OGF or LDN had no deleterious long-term repercussions and did not exacerbate EAE, but i) halted progression of disease, ii) reversed neurological deficits, and iii) prevented the onset of neurological dysfunction across a considerable span of time.

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⁎ Corresponding author at: Department of Neural and Behavioral Sciences, H109, The Milton S. Hershey Medical Center, 500 University Drive, Room C3729, Hershey, PA 17033, USA. Fax: +1 717 531 5003.
E-mail address: isz1@psu.edu (I.S. Zagon).

Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; OGF, opioid growth factor; OGFr, opioid growth factor receptor; LDN, low dose naltrexone; MOG, myelin oligodendrocyte glycoprotein; NTX, naltrexone; CFA, complete Freund’s adjuvant; GFAP, glial fibrillary acidic protein

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1. Introduction

Multiple sclerosis (MS) is a chronic and debilitating autoimmune disease of the central nervous system (CNS) that affects 400,000 people in the United States and 2 million individuals worldwide (Rizvi and Agius, 2004; Forte et al., 2007). While the etiology of MS is unclear, disease manifestation involves inflammation, demyelination, and axonal damage in the CNS (Bennett and Stuve, 2009; Weiner, 2009; Van der Walt et al., 2010). The first neurologic episode is termed “clinically isolated syndrome” (CIS), with an estimated 35,000 patients presenting each year, and 80% or more converting from CIS to clinically definite MS (Pandey and Lublin, 2009). The proliferation of immune cells and the production of myelin-specific antibodies at the site of active CNS lesions suggest that MS involves a dysregulation of the immune system, particularly T and B cells (Ercolini and Miller, 2006). Treatment/prevention/reparative of the neurodegeneration associated with the disease, particularly effective, inexpensive, non-toxic, and preferably orally delivered treatments, are needed.

The novel finding that endogenous opioids are biological factors that target growth was first reported in 1983 (Zagon and McLaughlin, 1983a,b). Using an innovative model that employed both an intermittent and continuous daily opioid receptor blockade with a low and high dose, respectively, of the opioid receptor antagonist naltrexone (NTX), native opioid peptides were hypothesized to function as tonically active inhibitory molecules for cell replication in a receptor-mediated manner. The duration of opioid receptor blockade proved critical in this concept (Zagon and McLaughlin, 1984; 1989a,b). NTX administration results in a compensatory upregulation of endogenous opioids and opioid receptors, and interaction of these elements takes place provided that there is a sufficient interval after NTX is no longer bound to a receptor. Hence, a low dose of NTX (LDN) can inhibit growth when NTX is no longer present after an initial 4- to 6-h period, leaving a window of 18–20 h for interfacing of the elevated opioids and opioid receptors that produces an exaggerated response (e.g., inhibition of cell replication). Subsequent studies (Zagon and McLaughlin, 1989a,b; 1991) revealed the endogenous opioid serving to tonically regulate cell proliferation through an inhibitory pathway was the pentapeptide [Met⁵]-enkephalin, termed opioid growth factor (OGF) to distinguish its distribution (neural and non-neural) and function (growth) from its original characterization as a neurotransmitter. The growth related function of OGF was found to be mediated by the non-classical opioid receptor, OGFr (originally termed zeta; Zagon et al., 1989, 2000), and the mechanism of OGF–OGFr action involves upregulating the cyclin-dependent inhibitory pathway and delaying the G1-S phase of the cell cycle (Chen et al., 2007, 2009a).

A series of reports (Zagon et al., 2009, 2010) reveal that endogenous opioids have a neuroprotective effect on encephalomyelitis (EAE), a prototype used to study MS in animals. Exposure to OGF interferes with autoimmune events and suppresses the onset and progression of EAE. Similarly, daily treatment with LDN has marked anti-EAE activity. However, exposure to a daily high dosage of NTX (HDN) that is continuously present for 24 h did not change the course of EAE expression, suggesting that opioid-receptor interaction is critical in modulating disease processes.

These earlier preclinical studies have focused on a short window of evaluation in terms of the effects of OGF and LDN on EAE, with behavioral observations limited to 30 days and neuropathology assessed after 20 days of treatment. This raises the important question of the whether OGF and/or LDN action is(are) short-term and diminish(es) after a longer course of treatment, or if the modulations of myelin oligodendrocyte glycoprotein (MOG)-induced EAE by one or both of these treatments are sustained. In this study, mice were given injections of MOG and received daily injections of the opioid antagonist OGF, or LDN to invoke an intermittent opioid receptor blockade, beginning at the time of EAE induction. Outcome measures included analysis of behavior and incidence of remission across a 60-day period, as well as neuropathological assessment at 10, 30, and 60 days.

2. Results

2.1. General observations

No lesions or ulcerations at the injection sites were observed in any animal. Behavioral abnormalities were not noted in any group for the first week following MOG injections. There were no differences in body weight between any MOG-injected group, and no deaths were noted. Control+Vehicle, as well as Normal mice, did not present with behavioral signs of EAE.

2.2. LDN and OGF treatment and EAE: behavioral assessment

Daily administration of LDN and OGF significantly decreased disease scores compared to MOG+Vehicle mice (Fig. 1A). Average disease scores of MOG+LDN and MOG+OGF mice remained markedly lower than MOG+Vehicle mice from days 15–39 and 50–60. The maximum average disease score reached by the MOG+Vehicle group was 2.0, and this score was observed on day 24. Conversely, MOG+LDN and MOG+OGF groups did not reach their maximum average disease scores of 1.3 and 1.1 until days 40 and 45, respectively (Fig. 1A).

All 45 animals injected with MOG that received Vehicle expressed behavioral signs of EAE (disease score ≥1) by day 22. However, only 68% of the MOG+LDN mice, and 63% of the MOG+OGF mice, ever presented with behavioral symptoms of EAE (Fig. 1B). The maximum disease incidence for the MOG+LDN and MOG+OGF groups was reached on days 30 and 45, respectively (Fig. 1B).

The first appearance of clinical disease was day 12 for the MOG+Vehicle and MOG+LDN groups, and day 13 for the MOG+OGF group. The average day of disease onset was delayed by 2 and 5 days for the MOG+OGF and MOG+LDN groups, respectively, in contrast to that for the MOG+Vehicle group (Fig. 1C). The mean maximal severity scores for the MOG+Vehicle group (1.9±0.1) were reduced by 26% and 37% for the MOG+LDN and MOG+OGF groups, respectively, and these differences were statistically significant (at least p<0.05). Calculation of the disease index over the entire course of the study (i.e.,
60 days) revealed a 2.6- and 3.2-fold reduction for the MOG+LDN and MOG+OGF groups, respectively, compared to the MOG+Vehicle group (Fig. 1D).

Analysis of individual disease scores at specific time points throughout the 60 days showed significant differences between EAE scores in the MOG+Vehicle and MOG+LDN or MOG+OGF-treated groups (Fig. 2). Although no differences in the proportion of mice displaying a limp tail (score=1) or hind limb paralysis (score=3) were recorded between groups, markedly more animals in the MOG+LDN and MOG+OGF groups had scores of 0 (no disease) on days 30 and 60. Moreover, with respect to animals displaying a disease score of 2 (wobbly gait), there was a 42%–72% reduction for the MOG+LDN and MOG+OGF groups, respectively, in contrast to the MOG+Vehicle group.

Over the 60 day course of the experiment, mice in both the MOG+LDN and MOG+OGF groups had 23% and 42%, respectively, more remissions than animals in the MOG+Vehicle group (7%) (Fig. 3).

2.3. LDN and OGF treatment and EAE: neuropathological assessment

Because not all of the mice injected with MOG+LDN or MOG+OGF developed behavioral signs, MOG+LDN and MOG+OGF-treated animals were further separated into groups that either expressed behavioral signs of EAE (MOG+LDN/EAE⁺, MOG+OGF/EAE⁺) or those that did not (MOG+LDN/EAE⁻, MOG+OGF/EAE⁻) for morphological assessment at 30 and 60 days.
2.3.1. Astrocyte activation

The number of reactive astrocytes on days 10, 30, and 60 post-EAE induction revealed activation of astrocytes for all animals (Fig. 4). At all time points examined, MOG+Vehicle mice had significantly more astrocyte activation in the lumbar region of the spinal cord compared to Control+Vehicle mice. Astrocyte activation was markedly reduced by 1.7- and 1.4-fold in MOG+LDN and MOG+OGF mice, respectively, compared to MOG+Vehicle mice on day 10. All MOG-injected groups had at least 1.6-fold more activated astrocytes compared to Control+Vehicle mice on day 30. MOG+LDN/EAE+ and MOG+LDN/EAE− animals had activated astrocyte counts that were comparable, but both LDN groups had notably fewer activated astrocytes than MOG+Vehicle mice. MOG+OGF/EAE− mice had a reduction in activated astrocytes with respect to animals of the MOG+Vehicle and MOG+OGF/EAE+ groups on day 30. On day 60, MOG+Vehicle astrocyte counts were 1.9-fold higher than Control+Vehicle counts. MOG+LDN/EAE+ and MOG+LDN/EAE− astrocyte counts were 1.8- and 1.4-fold greater, respectively, than Control+Vehicle counts. On day 60, MOG+OGF/EAE− counts were 1.8-fold higher than Control+Vehicle counts, while MOG+OGF/EAE+ did not differ compared to Control+Vehicle astrocytes. Day 60 MOG+OGF/EAE− mice had fewer activated astrocytes in contrast to animals in the MOG+Vehicle group.

2.3.2. Demyelination

Control+Vehicle animals did not exhibit signs of spinal cord demyelination at any of the 2 time points examined (Fig. 5). No demyelination was detected in the spinal cord of any MOG-injected animal at 10 days post-EAE induction. On day 30, 50% or more of the mice receiving MOG had at least one demyelinated quadrant. The MOG+Vehicle, MOG+LDN/EAE+, MOG+OGF/EAE+, and MOG+OGF/EAE− groups on day 30 had significantly more mice with demyelinated quadrants than the Control+Vehicle group, while animals in the MOG+LDN/EAE− group did not differ from the Control+Vehicle cohort. All groups except for MOG+OGF/EAE− mice had a marked increase in the number of demyelinated quadrants of the lumbar region of the spinal cord in comparison to the Control+Vehicle group on day 30. MOG-

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**Fig. 2** – The distribution of animals receiving disease scores of 0 (normal), 1 (limp tail), 2 (wobbly gait), and 3 (hind limb paralysis) at 30 and 60 days post-EAE induction. MOG+Vehicle, n = 11–45; MOG+LDN, n = 11–44; MOG+OGF, n = 10–38. Significantly different from MOG+Vehicle at p < 0.001 (**).  

**Fig. 3** – The percentage of animals with EAE that exhibited remission. MOG+Vehicle, n = 45; MOG+LDN, n = 44; MOG+OGF, n = 38. Significantly different from MOG+Vehicle at p < 0.001 (**) using chi square analysis.
Fig. 4 – The number of activated astrocytes in the lumbar region of the spinal cord in animals at 10 \( (n=3-4/\text{group}) \), 30 \( (n=3-7/\text{group}) \), and 60 \( (n=2-6/\text{group}) \) days post-EAE induction. On days 30 and 60, MOG+LDN and MOG+OGF-treated animals were separated into groups that either expressed behavioral signs of EAE (MOG+LDN/EAE\(^+\), MOG+OGF/EAE\(^+\)) or those that did not (MOG+LDN/EAE\(^-\), MOG+OGF/EAE\(^-\)). Photomicrographs show resting (Control+Vehicle) and activated (MOG+Vehicle) astrocytes. Bar=20 \( \mu \text{m} \). Data represent the percent of activated astrocytes relative to mice of the Control+Vehicle group. Significantly different from Control+Vehicle at \( p<0.05 \) (*), \( p<0.01 \) (**), or \( p<0.001 \) (***) . Significantly different from MOG+Vehicle at \( p<0.05 \) (+), \( p<0.01 \) (++) , or \( p<0.001 \) (+++). Significantly different from MOG+OGF/EAE\(^+\) at \( p<0.05 \) (♦).
injected animals receiving OGF and not displaying behavioral abnormalities (MOG+OGF/EAE) did not differ in the number of quadrants with demyelination compared to the Control+Vehicle group. On day 60, all groups that expressed behavioral signs of EAE (MOG+LDN/EAE+, MOG+OGF/EAE+) or those that did not (MOG+LDN/EAE−, MOG+OGF/EAE−) had spinal cord demyelination, whereas the spinal cords from animals with no clinical expression of EAE had no demyelination. The severity of demyelination as measured by the average number of demyelinated quadrants per spinal cord in any MOG-injected group did not differ from each other or the Control+Vehicle group. In fact, none of the mice in these MOG-injected groups had more than 1 quadrant expressing demyelination.

2.3.3. Neuronal damage
Examination of sections stained with SMI-32 revealed that both Control+Vehicle and MOG-injected mice had positive
neurons, an indicator of neuronal damage. Although some groups of MOG-injected mice had up to a 3-fold change in the number of neurons that were positive for SMI-32 compared to the Control+Vehicle group, no statistical differences were recorded between any of these groups or with animals in the Control+Vehicle group (Fig. 6).

3. Discussion

This study shows that modulation of opioid peptide-opioid receptor interactions by either daily administration of a native opioid peptide, OGF, or intermittent opioid receptor blockade with LDN, beginning at the time of EAE induction has long term repercussions in repressing the appearance and progression of disease. Over 60% of the MOG injected mice receiving OGF or LDN ever presented with behavioral symptoms of EAE and, in those mice that did, the severity of behavioral abnormality was markedly reduced from MOG-injected animals treated with Vehicle. Treatment with LDN or OGF resulted in 3- and 6-fold, respectively, more animals exhibiting remission of the behavioral symptoms of EAE in comparison to mice receiving MOG and Vehicle. Neuropathological examination revealed that although no behavioral signs were noted in any MOG-injected group at 10 days, the lumbar region of the spinal cord of all groups had activated astrocytes and neuronal damage, but no demyelination. Although demyelination was most severe at 30 days post-MOG injection in all groups, and was markedly reduced at 60 days, MOG-injected mice treated with OGF or LDN and not displaying behavioral symptoms of disease had no demyelination. However, activated astrocytes in OGF and LDN treated mice were notably reduced at 10 and 30 days from the MOG+Vehicle group, but this measure was fairly comparable in each MOG-injected group by 60 days. Neuronal damage was noted in all MOG-injected groups on days 30 and 60, with no distinct trend noted between or within groups. Interestingly, and keeping in mind the limited number of neuropathological measures performed, at no time was there a structural outcome in mice given OGF or LDN that could be correlated with a positive or negative display of behavioral symptoms. Taken as a whole, these studies extend our earlier reports (Zagon et al., 2009, 2010) on OGF and LDN as to EAE that were limited to observations of less than a month, and show that treatment with either agent not only has no deleterious long-term effects or an exacerbation of EAE, but can i) halt progression of the disease, ii) reverse neurological deficits, and iii) prevent the onset of neurological disorders across a considerable span of time.

In view of the extensive knowledge about the mechanism underlying OGF action with respect to cell proliferation, we can now place this information together with its effects on the expression of EAE recorded herein to suggest a hypothesis for the efficacy of this peptide. OGF is an autocrine and paracrine factor that enters cells by way of active transport (Zagon et al., 1991) that enters cells by way of active transport anddisciplinary transport dependent on nuclear localization signals encoded in OGFr (Cheng et al., 2009b), as well as karyopherin β and Ran (Cheng et al., 2010b) which serve in guidance between the cytoplasm and nucleus. OGF-OGFr enters the nucleus, increases p16 and/or p21 cyclin-dependent inhibitory kinases (Cheng et al., 2007, 2009a), and delays the G1-S phase of the cell cycle (Cheng et al., 2007, 2009a). With regard to EAE and MS, both involve autoimmune diseases that are widely thought to be mediated by T cell-mediated immunity (Bennett and Stuve, 2009; Weiner, 2009), although there is evidence for humoral immunity in the disease process: an antigen-driven B-cell response (Bennett and Stuve, 2009; Weiner, 2009). Moreover, T and B cells depend on proliferation for response, and OGF has been reported to depress T and B cell proliferation (Zagon et al., 2011a,b) by an OGFr dependent inhibitory pathway involving p16 and p21 (Zagon et al., 2011a,b). Thus, in the present context it could be postulated that the OGF–OGFr axis has a prolonged downregulation of the proliferation of immune cells responsible for the early events in inflammation. This immunosuppression exerted through native biological channels in turn reduces astrocyte activation and demyelination but not neuronal damage, with the final outcome being a diminishment in neurological dysfunction.

The mechanism underlying LDN’s activity in depressing the expression of EAE requires elucidation. NTX is a general opioid antagonist known to block both classical (µ, δ, κ) and non-classical (OGFr) opioid receptors (Leslie, 1987). In an earlier study, intermittent opioid receptor blockade with a low dose of NTX (i.e., LDN) was discovered to repress the expression of EAE (Zagon et al., 2009). However, continuous daily opioid receptor blockade with NTX had no effect on the course of EAE (Zagon et al., 2009). These results indicated that the duration of opioid receptor blockade is critical in regulating the expression of EAE, but that the tonic regulatory property of peptide-receptor interaction was absent. LDN is known to upregulate endogenous opioids and opioid receptors in an effort to compensate for the blockade of peptide from receptors and, acting in the interval after NTX is no longer present, can downregulate cell proliferative processes (Zagon and McLaughlin, 1995). Since endogenous opioids are growth regulators, it may be conjectured that LDN reduces cell proliferation – particularly immune cells (e.g., T and B lymphocytes) – by an exaggerated response to one or more of these endogenous opioids interfacing with one or more opioid receptors. Pursuing this further, in a series of reports (Zagon et al., 2011a,b) OGF was the only endogenous opioid peptide to have an effect on T and B cell replication. Using siRNA technology to diminish classical (µ, δ, and κ) and non-classical (OGFr) opioid receptors in the face of OGF action in these cells, it was discovered that OGF only interacts with one opioid receptor: OGFr. Thus, OGF depresses T and B cell proliferation in cells with a knockdown of either µ, δ, or κ opioid receptors, but has a negligible effect in cells transfected with siRNA for OGFr. Thus, it may be that the OGF–OGFr axis is the common denominator of both OGF and LDN activity in EAE.

With the potential importance of opioids as a means of neuroprotection with respect to MS, a number of intriguing observations in patients with MS may be correlated. β-endorphin has been reported to be in the serum and CSF of individuals with MS, and is produced by activated lymphocytes (Gironi et al., 2000).
Fig. 6 – The number of damaged neurons in the lumbar region of the spinal cord in animals at 10 (n=3–4/group), 30 (n=2–5/group), and 60 (n=2–6/group) days post-EAE induction. On days 30 and 60, MOG+LDN and MOG+OGF-treated animals were separated into groups that either expressed behavioral signs of EAE (MOG+LDN/EAE+, MOG+OGF/EAE+) or those that did not (MOG+LDN/EAE−, MOG+OGF/EAE−). Photomicrographs show spinal cord sections taken from animals with no neuronal damage (Control+Vehicle) and neuronal damage (MOG+Vehicle). Data represent the percent of damaged neurons relative to control+Vehicle mice. Bar=20 μm.
Gironi et al. (2000) have reported a reduction in β-endorphin levels in peripheral blood mononuclear cells (PBMCs) from patients with clinically inactive MS, but demonstrated an increase in PBMCs from patients with clinical relapse. No change in another opioid peptide, prodynorphin, has been detected in the blood of patients with MS (Roy et al., 1994). Jankovic (1991) has reported that treatment with [Met5]-enkephalin had a beneficial effect on 13 patients with chronic severe progressive MS. MS patients usually go into remission during pregnancy, and fewer relapses are observed during this period (Csontos et al., 1979). Interestingly, a marked rise in endogenous opioids occurs during pregnancy (Csontos et al., 1979). A role for opioid peptides in the development and progression of MS has been suggested by other studies which demonstrate that proteases associated with degradation of opioid peptides are elevated in MS. For example, Ziaber et al. (1999, 2000) reported an increase in CD10 (neutral endopeptidase-NEP; EC 3.4.24.11) and CD13 (aminopeptidase N; AP-N, EC 3.4.11.2) in MS patients during the course of exacerbation and chronic MS, but low expression of these molecules during remission. Because these ectoenzymes are enkephalin-degrading (Dass and Mahalakshmi, 1996) they may lead one to suggest that enkephalins promoting cell proliferation would be decreased and thereby lead to an increase in both T cell proliferation and the development of MS. Finally, a genome-wide study has identified MS susceptibility loci on chromosomes 12q13–14 and 20q13 (The Australia and New Zealand Sclerosis Genetics Consortium, 2009). One opioid receptor – the nonclassical OGF which is the receptor for OGF – is indeed located at 2q13.3 (Zagon et al., 2000). Together, the above findings have established an important link between opioid peptides and MS.

Based on our preclinical results showing that OGF and LDN can change the course of EAE, clinical trials with these drugs need to be considered. Both OGF and LDN have proven safety and demonstrate efficacy in clinical trials for pancreatic cancer (Smith et al., 2004, 2010) and Crohn’s disease (Smith et al., 2007), respectively. In fact, at least with LDN, some clinical information already exists in patients with MS. Gironi and colleagues (2008) have reported on the safety of LDN in patients with primary progressive MS, while Cree and coworkers (2010) found that LDN was well tolerated and serious adverse health issues did not occur in patients with clinically definite MS. Using LDN in animals with relapsing-remitting and secondary progressive MS, Sharaafiddinzadeh et al. (2010) reported that LDN was safe but a longer duration trial was needed to determine efficacy. Thus, it appears that transition from bench to bedside is feasible when designing studies using OGF or LDN for the treatment of MS. Given the present results, patients with CIS – and perhaps clinically definite MS (e.g., relapsing-remitting) – may greatly benefit from treatment with OGF or LDN.

4. **Experimental procedures**

4.1. **Animals and induction of experimental autoimmune encephalomyelitis**

C57BL/6 female mice (7–10 weeks) were purchased from Harlan Laboratories (Indianapolis, IN) and maintained at the Penn State Hershey Medical Center, with food and water provided ad libitum. All experiments were conducted in accordance with the NIH guidelines on animal care and were approved by the Penn State Hershey Institutional Animal Care and Use Committee.

EAE was induced in mice following earlier reports (Suen et al., 1997; Zagon et al., 2009, 2010). In brief, mice were given a total of 900 μg of mouse MOG35–55 (Penn State College of Medicine Core Facility, 99% purity) dissolved in phosphate-buffered saline (PBS) and emulsified in complete Freund’s adjuvant (CFA, DIFCO Laboratories, Lawrence, KS), supplemented with 500 μg heat-inactivated Mycobacterium tuberculosis (DIFCO Laboratories) through a series of injections. The injections were divided equally between the left and right flanks. Intraperitoneal injections of 500 ng pertussis toxin (List Biological Laboratory, Campbell, CA) dissolved in 200 μl PBS were administered on days 0 and 2. Animals injected with CFA, pertussis, and PBS served as Control+Vehicle animals. Animals that were not given CFA, pertussis toxin, or MOG peptide were considered Normal. Five individual experiments were conducted, with 5–10 animals/group/experiment. Data for behavioral analysis of mice from days 1 to 30 included two experiments with OGF and three experiments with LDN that were previously published (Zagon et al., 2009, 2010).

4.2. **Drug treatment**

Daily intraperitoneal injections of 0.2 ml LDN (0.1 mg/kg NTX), OGF (10 mg/kg), or Vehicle (PBS) began on the day of induction (day 0). Injections were given at the same time each day (approximately 1300 h).

4.3. **Behavioral evaluation**

Confirmation of EAE was made by daily assignment of disease score based on previous studies (Encinas et al., 1996; Suen et al., 1997; Zagon et al., 2009, 2010). Disease severity was evaluated on a scale of 0–5: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 2.5, single hind limb paralysis; 3, complete hind limb paralysis; 4, hind and fore limb paralysis; 5, death. Animals were assigned disease scores in a masked fashion at the same time each day, and a disease score ≥1 for two consecutive days was required for confirmation of EAE. Mean maximal severity score was calculated according to Milicevic et al. (2003) and represented the mean of the maximal disease score that each mouse in a group developed over the course of the experiment. Remission was noted when an animal with a disease score ≥1 returned to a disease score of 0 for two consecutive days. Disease index, the mean daily clinical score for all animals in a treatment group divided by the average day of disease onset and multiplied by 100, was calculated according to Suen et al. (1997). For those animals in MOG groups that did not display behavioral symptoms of EAE before the time of sacrifice, day of disease onset was assigned as 1 day after the experiment was terminated. The maximum average disease score for each group, and the day on which it occurred, was noted from the data on the disease index.

4.4. **Histopathology**

At 10, 30, and 60 days of treatment, mice were anesthetized with a cocktail (0.1 ml) of ketamine (30 mg/kg), xylazine (5 mg/kg),...
and acepromazine (2 mg/kg), and perfused transcardially with 10% neutral buffered formalin. Tissues were fixed for 48 h, and spinal cords dissected. Matched paraffin sections (10 μm) of the lumbar region (L4-L6) (Sidman, 1979), were stained with (i) a polyclonal antibody to GFAP (1:500 dilution, DAKO, Carpinteria, CA) to identify activated astrocytes (stellate-shaped cells with hypertrophic processes), (ii) a mouse monoclonal antibody to SMI-32 (1:500 dilution, Covance, Princeton, NJ) to detect neuronal damage as indicated by the presence of non-phosphorylated neurofilament H protein, and (iii) Luxol Fast Blue (Roboz Surgical Instrument Co., Washington, DC)-Cresyl Violet (Sigma, St. Louis, MO) to assess myelin. Sections stained with secondary antibody only served as controls. At least two nonconsecutive sections/animal, with 2–7 animals/group/timepoint, were assessed for each measure. All measures were recorded by an observer masked to identification of the treatment group.

To assess demyelination, spinal cord sections were divided into quadrants by a horizontal and a vertical line that intersected through the central canal. The number of quadrants with demyelination (the absence of Luxol Blue staining) were counted according to Tsunoda et al. (2001) and MacNamara et al. (2005).

The number of reactive astrocytes (i.e., GFAP positive) (Bannerman et al., 2007; Stichel and Luebbert, 2007) or damaged neurons (i.e., SMI-32 positive) per grid (0.053 mm2 grid at 400×) was counted with an Olympus BH-2 microscope according to Bannerman and Hahn (2007) and Bannerman et al. (2007). Three grids were counted per spinal cord section.

4.5. Statistical analysis

Behavioral and histopathology data were analyzed using Student’s t-test or one-way analysis of variance (ANOVA), with subsequent comparisons made with Newman Keul’s post-tests (GraphPad Prism Software, La Jolla CA). Some data were analyzed by chi-square analysis (GraphPad Prism). p Values less than 0.05 were considered statistically significant.

Acknowledgments

This work was supported in part by the National Multiple Sclerosis Society, the Paul K. and Anna E. Shockey Family Foundation, and the Zagon/Kostel families.

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