Published in final edited form as:

J Neuroimmunol. 2011 April ; 233(1-2): 90–96. doi:10.1016/j.jneuroim.2010.12.002.

Lipoic Acid Decreases Inflammation and Confers Neuroprotection in Experimental Autoimmune Optic Neuritis

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Abstract

Lipoic acid (LA) is an antioxidant that is effective in treating experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS). C57BL/6 mice with EAE develop experimental autoimmune optic neuritis (EAON), which models acute optic neuritis in humans. Here we determined whether LA is therapeutically effective in EAON. We immunized C57BL/6 mice with MOG 35–55 peptide. Mice received either daily subcutaneous injections of LA (100 mg/kg) or saline in early or late suppression paradigms. In the early suppression paradigm, optic nerve cross sections showed $14.9 \pm 3.8\%$ (mean $\pm$ SEM) damage in mice receiving saline ($n = 7$) and $2.0 \pm 0.4\%$ damage in mice given LA ($n = 7$, $p = 0.001$). In the late suppression paradigm, optic nerve sections showed $24.6 \pm 3.5\%$ damage in mice treated with saline ($n = 7$) and $8.4 \pm 2.5\%$ in mice treated with LA ($n = 7$, $p = 0.004$). Thus a dramatic reduction in axonal injury was seen after LA administration in both experimental paradigms. Compared with saline treated mice with EAON, optic nerves from mice receiving LA had significantly fewer CD4+ and CD11b+ cells in both paradigms. This study provides a rationale for investigating the therapeutic efficacy of LA in acute optic neuritis in humans.

Keywords

lipoic acid; optic neuritis; EAON; multiple sclerosis; neuroprotection

1. Introduction

Acute optic neuritis (AON) can occur in isolation or as a manifestation of multiple sclerosis (MS) and results in demyelination and variable axonal loss in the optic nerve. While high dose methylprednisolone can speed up recovery from AON, there are presently no treatments to prevent axonal damage in AON (Abou Zeid and Bhatti, 2008; Shams and Plant, 2009). Experimental autoimmune encephalomyelitis (EAE) in mice is used as a model

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system for MS. Experimental autoimmune optic neuritis (EAON) frequently occurs in mice with EAE and models AON (Shao et al., 2004; Shindler et al., 2006). EAON models have been used as a standard system to study the pathogenesis and treatment of AON (Maier et al., 2006; Qi et al., 2007; Sattler et al., 2004).

Degeneration of axons causes impairment of vision following AON and permanent disability in MS. The mechanisms of axon loss in both are being investigated. Changes in ion channels, cytoskeletal pathology, reduced ATP production, and decreased axonal transport all may contribute to axonal degeneration (Dutta et al., 2006; Young et al., 2008).

Mitochondrial dysfunction and oxidative stress have come into focus recently and are believed to play a major role in the degeneration of axons in inflammatory disorders of the CNS, such as AON and MS (Dutta et al., 2006; Forte et al., 2007; Guy, 2008; Kalman and Leist, 2003; Smith and Lassmann, 2002).

Lipoic acid (LA) is a natural antioxidant that suppresses and treats EAE (Chaudhary et al., 2006; Marracci et al., 2002; Morini et al., 2004; Schreibelt et al., 2006). LA mediates its effect by inhibiting T cell migration into the CNS, decreasing endothelial cell adhesion molecule expression and causing a reduction in MMP-9 production. Recently, we found that LA stimulates cAMP production via G protein coupled prostaglandin EP2 and EP4 receptors on immune cells (Salinthon et al., 2008; Schillace et al., 2007). cAMP is an important secondary messenger involved in signal transduction pathways (Kamenetsky et al., 2006) and cAMP regulatory pathways play a role in immunoregulation and neuroprotection. Thus, cAMP might be vital to the therapeutic effects of LA (Silveira and Linden, 2006). Protection against loss of axons is central to development of therapies for AON and MS. In this study we assess the effects of LA in EAON for the first time and demonstrate that LA significantly reduces axonal injury in EAON.

2. Materials and Methods

2.1. EAON induction and assessment

C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) female mice were immunized with 200 µg of MOG 35–55 peptide in complete Freund's adjuvant containing 400 µg of *Mycobacterium tuberculosis* per mouse by subcutaneous (sc) injection (a total volume of 0.2ml). Pertussis toxin was administered by intraperitoneal (ip) injection at day 0 (75 ng per mouse) and day 2 (200 ng per mouse) after immunization. Mice were scored daily for EAE as previously described (Forte et al., 2007). There are no behavioral manifestations of EAON but all C57BL/6 mice with clinical signs of EAE have histologic evidence of EAON.

2.2. Lipoic acid administration in mice

LA was obtained from Sigma, MO. LA (50 mg) was weighed and resuspended in 0.35 ml 2N NaOH. Sterile water (4.5ml) was added and titrated with approximately 0.05 ml 10N HCl or until LA barely precipitated. pH was adjusted to 7.2 – 7.4. The solution was filter sterilized through a 0.2 µm low protein binding syringe filter and frozen in aliquots until used. The frozen solution was thawed at 37°C and diluted as necessary in sterile saline prior to administration. 0.4 ml LA (5 mg/ml) was administered by sc injection (100 mg/kg). Equal

volume of a normal saline solution was used as a vehicle-control. Mice received either daily sc injections of LA (100 mg/kg) or saline beginning on Day 8 after immunization (early suppression paradigm) or day 13 post-immunization after the first mouse displayed signs of EAE (late suppression paradigm). Mice were sacrificed 21 days after immunization for the early suppression paradigm and 24 days after immunization for late suppression paradigm. Naïve C57BL/6 mice were used as histologic controls. Optic nerves were processed for histologic measurement of axonal damage and demyelination (toluidine blue staining, % area damage). Optic nerves were also used to measure inflammatory cells (immunofluorescence, thresholding, % area stained).

2.3. Quantitative Morphological Determination of Percentage of Tissue Damage

Mice were deeply anesthetized with isoflurane, heparinized, and perfused with ice cold 4% paraformaldehyde. The right optic nerve was dissected out and processed for immunohistochemical staining, as described below. The left optic nerve was dissected out and fixed in 5% glutaraldehyde and processed for quantitative morphology. The left optic nerve tissue samples were post-fixed with 1% osmium, dehydrated in ethanol, and embedded in plastic. Semi-thin sections (0.5 µm) were stained with toluidine blue. Photomontages of the entire optic nerve cross section were made, areas containing damaged axons were manually circled at 100× and measured by using BIOQUANT software (ver 8.12, Nashville, TN) as has been done previously in spinal cord tissue in EAE (Forte et al., 2007). Tissue sections were analyzed blinded to treatment status. The cross-sectional area was determined by outlining the entire optic nerve. Degenerating axons with abnormal morphology, myelin ovoids, unstable myelin, area with absence of axons and microcyts were marked as damaged. The damaged area was added together and % damaged area was determined for each optic nerve using the following formula: area of damage/total cross-sectional area × 100%.

Thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined using a Phillips Morgagni 268 transmission electron microscope. Images were captured with a MEGAPLUS Camera ES 4.0, and AMTV540 imaging software.

2.4. Quantitative immunohistochemical studies

For quantitative immunofluorescent analyses of the right optic nerves, three 50-µm sections were randomly selected for antibody staining from each optic nerve. The sections were permeabilized, washed, blocked (0.5% fish skin gelatin/3% BSA) in PBS, and then incubated with primary antibody at 4°C overnight. After incubation in secondary antibody, sections were mounted in Prolong Gold antifade and imaged using laser scanning confocal microscope system (Olympus FluoView mounted on BX51 microscope, Melville, NY).

Primary antibodies that recognized CD4 (BD PharMingen, San Diego, CA; 1:25 dilution) and Mac-1 (CD11b; Leinco Technologies, St. Louis, MO; 1:75 dilution) were used to identify T cells and microglia/macrophage populations, respectively. Alexa Fluor 488 donkey anti-rat IgG (Invitrogen, Carlsbad, CA; A21208; 1:200 dilution) was used as the secondary antibody. To quantify immunofluorescence, sections were imaged (40× objective) on an Olympus confocal microscope. Twelve-bit images were captured at a resolution of

1024×1024 pixels using a UplanF1 40×/1.3 oil-immersion objective with FluoView 5.0 software. Data analyses were performed using MetaMorph software (Molecular Devices, CA). Negative controls were generated by omitting the primary antibody. An additional control comprised omission of both primary and secondary antibodies.

For quantitative image analysis primary antibodies and fluorophore conjugated secondary antibodies that have high specificity, low background, and lack of cross reactivity were chosen. For an experiment all the sections were stained simultaneously. Imaging on a laser scanning confocal microscope was standardized to use the entire dynamic range without saturation. Same settings of power, gain, and offset were used to maintain consistency for comparison between groups. Data analysis was done using MetaMorph software, v7.6.3; Molecular Devices, CA. Thresholding and intensity measurement methods of image analysis were used. Area was analyzed in about 20,000 sq microns. Image analyses were done blinded to treatment.

The average background fluorescence was determined from several areas containing unlabeled regions, naïve sections, and sections incubated in secondary antibody only. Thresholding enabled us to select ranges of pixel values that separate the objects under consideration from the background. The percentage of threshold area was compared between different groups. Similar methods have been used for quantification of CD4+ and CD11b+ cells in spinal cords of mice with EAE (Chaudhary et al., 2006; Forte et al., 2007).

2.5. Statistical Analyses

Statistical analyses were performed using the Mann–Whitney *U*-test. Statistical significance was defined as $p < 0.05$ for all analyses.

3. Results

We first determined the prevalence optic neuritis in C57BL/6 mice immunized with MOG 35–55 peptide to induce EAE before and after the onset of clinical EAE. Mice with EAON did not manifest clinical signs of visual impairment. In two separate experiments on C57BL/6 mice with EAE, we measured axonal injury 10 and 30 days after immunization; at 10 days after immunization, none of the mice had developed signs of EAE whereas at 30 days after immunization all of the mice had developed clinical signs of EAE. Optic nerves had an average area of damage of 7.3 ± 1.1 % ($n = 4$) 10 days after immunization when none of the mice had signs of EAE. At day 30 after immunization, we detected an area of damage of 38.2 ± 6.9 % ($n = 5$). Thus axonal injury occurs by 10 days after immunization before mice develop clinical EAE. In the early suppression paradigm, LA or saline were started eight days after immunization. In the late suppression paradigm as determined by clinical signs of EAE, LA or saline were started at day 13 after immunization; with this paradigm, LA was started after the onset of histologic optic neuritis and therefore could be considered a treatment for EAON.

To assess the effect of LA on optic nerves, we immunized C57BL/6 mice with MOG 35–55 peptide. The groups were divided randomly into early suppression (8 days after immunization) and late suppression (13 days after immunization) paradigms. None of the

mice in either paradigm receiving LA developed signs of EAE whereas all mice in both paradigms receiving saline developed EAE (Table 1, Fig 1), consistent with our previous results in SJL mice (Chaudhary et al., 2006; Marracci et al., 2002, Table 1, Fig 1). Mice were perfused at day 21 (early suppression paradigm) or day 24 (late suppression paradigm). Naïve mice were perfused as controls. Optic nerves were processed for morphometry and immunohistochemistry. All optic nerves from saline recipients had more inflammatory cells than observed in optic nerves for naïve mice, indicating that EAON was induced in all mice not receiving LA; all but one optic nerve from mice receiving LA had inflammatory cells albeit at a reduced level compared with those from mice receiving saline.

3.1. LA protects axons in optic nerve

Figure 2 illustrates the method of quantification of damage in optic nerves. In the early suppression paradigm, $14.9 \pm 3.8\%$ (mean $\pm$ SEM) of the cross-sectional area of optic nerves from mice receiving saline ($n = 7$) revealed damage compared with $2.0 \pm 0.4\%$ of the cross-sectional area of optic nerves of mice given LA ($n = 7$) ($p = 0.001$), representing an 87% reduction in tissue damage. In the late suppression paradigm, $24.6 \pm 3.5\%$ of the cross-sectional area of optic nerves from mice receiving saline ($n = 7$) contained damage compared with $8.4 \pm 2.5\%$ of the cross-sectional area of optic nerves from mice receiving LA ($n = 7$) ($p = 0.004$, Fig 3, 4, Table 2), a 66% reduction in injury. Importantly, the cross-sectional areas of optic nerves from mice receiving saline and LA did not differ significantly and did not account for any of the differences in percentage of area of damage (Table 2). In areas of damage in both paradigms, optic nerves from mice receiving saline had decompacted/unstable myelin characteristic of degenerated myelin (Fig 3, Fig 4 a,c) and many degenerating axons (Fig 4 a,c). Degenerating axons filled with debris were seen in saline treated optic nerves (Fig 4c). A few demyelinated axons (Fig 4 a,c) were observed in mice receiving saline. Increased cellularity that consisted of macrophages, microglia and astrocytes was detected in optic nerves from mice receiving saline (Figures 3 and 4). Microcysts, which most likely were remnants of myelin sheaths devoid of degenerated axons, were seen in optic nerves from saline recipient mice (Fig 3 a,c). These spaces were similar to those seen in the rat optic nerve by Cepurna and colleagues (Cepurna et al., 2005) and spinal cord white matter by Zoller et al. (Zoller et al., 2008). The optic nerves of LA recipients in the early suppression paradigm appeared similar to the optic nerves from naïve mice, and only occasional degenerating axons were observed in optic nerves from LA recipients in the late suppression paradigm (Figs 3, 4). The optic nerves of mice receiving LA had reduced cellularity as compared with saline treated optic nerves in both paradigms.

3.2. LA inhibits infiltration of inflammatory cells into optic nerve

Optic nerves from naïve mice did not have detectable CD4+ T cell staining; the % area with CD11b+ macrophage/microglia staining was 1.2 ± 0.18 (Table 2, Fig 5). In the early suppression paradigm, the % area occupied by CD4+ T cells was 1.6 ± 0.2 in mice receiving saline and 0.6 ± 0.1 in mice receiving LA ($p = 0.001$) (Table 2, Fig 5). The % area with CD11b+ macrophages/microglia in the early suppression paradigm was 6.9 ± 1.0 in mice that received saline and 2.4 ± 0.4 in mice that received LA ($p = 0.0004$) (Table 2, Fig 5). In the late suppression paradigm, LA also decreased CD4+ and CD11b+ immune cells in optic nerves. The % area occupied by CD4+ T cells in mice receiving LA was 1.2 ± 0.3 and in

mice receiving saline was 2.2 ± 0.3 ($p = 0.008$) (Table 2, Fig 5). CD11b+ macrophages/microglia occupied 5.7 ± 0.8 of the optic nerve area in the LA group and 8.6 ± 1.8 in the saline group (Table 2, Fig 5); this difference was not statistically significant. All optic nerves in saline groups (early and late suppression paradigms) showed inflammatory cells. One mouse receiving LA in the early suppression paradigm did not have any inflammatory cells. In all other mice, inflammatory cells were observed in the optic nerves of the LA groups (early and late suppression paradigms) above that of optic nerves from naïve mice but at a significantly lower level than that of saline groups.

4. Discussion

This is the first report demonstrating that LA is highly effective at reducing inflammation and protecting axons in EAON. LA decreased the number of CD4+ T cells and CD11b+ macrophages/microglia and significantly reduced axonal degeneration in both the early and late suppression paradigms. It is likely that the decrease in axonal injury associated with LA administration was secondary to the marked reduction in inflammatory cells, although it is possible that LA conferred some neuroprotection directly. It should be emphasized that axonal injury in EAON begins before the onset of clinical EAE (as early as day 10 after immunization) and the late suppression paradigm as defined by clinical EAE criteria probably represents a treatment paradigm for EAON. Thus, LA not only suppresses EAON but is also effective therapeutically.

EAON that occurs in association with mice developing EAE (Shao et al., 2004; Shindler et al., 2006) models AON in humans and has been used to assess other therapies for AON (Guo et al., 2009; Qi et al., 2007; Shindler et al., 2007). Shao et al. induced optic neuritis in about 63% of C57BL/6 mice immunized with MOG 35–55 peptide and found that optic neuritis affected both eyes (Shao et al., 2004). In our study, mice with EAON did not manifest clinically apparent signs of visual impairment. Although we did not functionally test vision in the mice, we are confident that EAON was induced in all the mice that received saline (as detected by inflammation in optic nerves in mice with EAE). Histopathology of optic nerves after immunization with CNS-specific myelin proteins have been studied previously (Jones et al., 1990; Potter and Bigazzi, 1992; Shindler et al., 2008; Wang et al., 2005). In addition, Wu et al. detected axonal loss in optic nerve at day 20 with MRI imaging and histologic studies (Wu et al., 2007). Thus multiple groups have established the histologic features of EAON in association with varying models of murine EAE.

We and others have previously shown that LA is effective at both suppressing and treating EAE (Marracci et al., 2002; Morini et al., 2004; Schreibelt et al., 2006). We previously studied the effects of LA on EAE in SJL mice, which follows a relapsing remitting course and thus clinically mimics relapsing MS. We demonstrated that LA was effective at treating relapsing EAE when initiated after the onset of clinical EAE in this model. LA also reduced inflammation and reduced demyelination and axonal loss in the relapsing EAE model in SJL mice (Marracci et al., 2002). In the present study we demonstrated that LA is effective at suppressing EAE in C57BL/6 mice immunized with MOG 35–55 peptide. EAE in this model is monophasic and the primary pathology is that of multifocal axonopathies with relatively little primary demyelination (Gold et al., 2006; Jones et al., 2008). While MOG

35–55 peptide induced EAE in C56BL/6 mice has clinical and pathologic features that differ from MS, it is a useful model for studying immune mediated axonal degeneration. EAON in C57BL/6 mice is monophasic and models AON in humans. The ability of LA to dramatically inhibit axonal injury in EAON is relevant to AON since axonal degeneration is important to long term visual impairment in AON.

The mechanism of action of LA in EAON and EAE are incompletely understood. Following immunization, LA does not prevent development of T cell responses against myelin antigens. It does reduce migration of inflammatory cells into the spinal cord and, as demonstrated here, into the optic nerves. These effects may be mediated by its ability to inhibit production of MMP-9 by T cells and adhesion molecules ICAM-1 and VCAM-1 (Chaudhary et al., 2006; Marracci et al., 2004). It has been postulated that LA exerts these effects via inhibition of activation of NFkappaB (Suzuki et al., 1992; Zhang and Frei, 2001). However this mechanism has not been demonstrated *in vivo* and *in vitro* required much high concentrations of LA than that observed in blood of mice receiving therapeutic doses of LA (Yadav et al., 2010). More recently, we have demonstrated in human CD4+ and NK cells that LA can stimulate production of the cAMP, which in turn can activate protein kinase A (PKA) (Salinthon et al., 2010). PKA is involved in activating a number of intracellular signaling pathways that can modulate immune responses (Salinthon et al., 2010). Importantly, these effects of LA are not dependent on the antioxidant effects of LA, which are mediated by its reduced form dihydrolipoic acid. We are currently investigating whether the therapeutic effects of LA in EAON and EAE are dependent on stimulation of cAMP and activation of PKA.

Based on the ability of LA to suppress and treat EAE, we have initiated early clinical trials of orally administered LA in MS subjects (Yadav et al., 2005; Yadav et al., 2010). These studies have shown that a dose of 1200 mg per day of LA is well tolerated and produces peak serum concentrations equivalent to those of mice receiving therapeutic doses of LA (Yadav et al., 2010). In addition, LA administered to MS subjects for two weeks compared with placebo was associated with a reduction in sICAM-1 and MMP-9 in the serum. Based on the results presented here coupled with early clinical studies in MS subjects, we believe orally administered LA warrants investigation as a treatment for AON.

Acknowledgments

Grant information

Department of Veterans Affairs Biomedical Laboratory Research and Development, NIH P30-NS06180

National Multiple Sclerosis Society Grants CA1055A, Laura Fund for Innovation in Multiple Sclerosis Research.

Nancy Davis Center Without Walls

This work was supported by the Biomedical Laboratory Research and Development Service, Department of Veterans Affairs, National Multiple Sclerosis Society Grants CA1055A, NIH project P30NS061800 (Neuroscience Imaging Center at OHSU), the Laura Fund for Innovation in Multiple Sclerosis Research and the Nancy Davis Center Without Walls. We would like to express our thanks to the Advanced Light Microscopy Facility @ The Jungers Center and Dr. Elaine Johnson, Casey Eye Institute for discussions on histology of the optic nerve.

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Fig 1.

LA suppresses EAE. Clinical EAE severity was scored following active immunization with MOG 35–55 peptide. Panel A shows the disease course in which the mice were given daily s.c. injections beginning 8 days after immunization (early suppression; $n=7$ for each group). Panel B shows the disease course of the mice given daily s.c. injections beginning 13 days after immunization (late suppression; $n=7$ for each group). Arrow indicates start of LA administration. Bars represent SEM for each time point.

Fig 2.

Quantification of damage in optic nerves. The cross-sectional area was determined by outlining the entire optic nerve (in blue). Degenerating axons with abnormal morphology, myelin ovoids, unstable myelin and microcysts were marked as damaged (red). Damaged areas were manually outlined at 100 $\times$ (shown in red). As shown, naïve optic nerves had no areas of damage. Optic nerves from mice with EAON receiving saline had extensive damage and those receiving LA had much less damage. The damaged area was added together and % damaged area was determined using the total cross-sectional area from each optic nerve as 100%. Optic nerves from a-naïve, b-saline recipient, c-LA recipient.

Fig 3.

LA protects axons in optic nerve. Toluidine blue stained optic nerve sections are shown in this figure. (a) Optic nerve from EAON mouse that was sacrificed 21 days after immunization and received saline from day 8. (b) Optic nerve from EAON mouse that received LA 100mg/kg per day from day 8 after immunization and sacrificed 21 days after immunization. (c) Optic nerve from EAON mouse that received saline from day 13 and was sacrificed at 24 days after immunization. (d) Optic nerve from a mouse that was treated with LA 100mg/kg from day 13 and sacrificed 24 days after immunization (e) Optic nerve cross

section from a naïve mouse. Optic nerves from mice receiving saline reveal reduction in axon density, many degenerating axons within unstable myelin sheaths, myelin ovoids, microcysts, infiltrating mononuclear cells, and astrocytic gliosis. Optic nerves from mice receiving LA show normal axon density and only scattered degenerating axons. Scale bar is 20µm.

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Fig 4.

Electron micrographs of optic nerve cross sections. The images are taken at 4400 $\times$ magnification. (a) Optic nerve from EAON mouse that was sacrificed 21 days after immunization and received saline from day 8. (b) Optic nerve from EAON mouse that received LA 100mg/kg per day from day 8 after immunization and sacrificed 21 days after immunization. (c) Optic nerve from EAON mouse that received saline from day 13 and was sacrificed at 24 days after immunization (d) Optic nerve from mouse that was treated with LA 100mg/kg from day 13 and sacrificed 24 days after immunization. (e) Optic nerve cross section from a naïve mouse. Arrows show the following examples of pathology seen in EAON: 1) axon engulfed by macrophages, 2) degenerating axons, 3) demyelinated axon, 4) cellular debris in an axon. Arrow heads show astrocytic processes. Many degenerating axons with unstable myelin are apparent in optic nerve from the mice receiving saline (a, c). Axons are well preserved in optic nerves from mice receiving LA (b,d). Scale bar is 2 μ m.

Fig 5.

LA decreases inflammation. Anti-CD4 (top) and anti-CD11b (bottom) immunostained optic nerve sections. (a, d) Optic nerve from EAON mouse that received saline from day 8. (b, e) Optic nerve from EAON mouse that received LA 100mg/kg per day from day 8 after immunization. Mice were sacrificed 21 days after immunization. (c, f) Optic nerve cross section from a naïve mouse. Note increased inflammatory infiltrates (CD4+ and CD11b+ cells) in mice that were administered with saline prior to disease onset. Much reduced staining occurred in optic nerves of mice that were administered LA. Naïve mice have very little CD4+ staining and some CD11b+ cells, which were most likely microglia. A similar staining was observed in late suppression paradigm. Scale bar is 50µm.

Table 1

EAE and EAON after MOG 35–55 peptide immunization

Groups	Number of mice	Average clinical score (last day)	Average clinical scores (total)	Mice with EAE	Histological abnormalities in ON (toluidine blue staining)	Inflammation
Early suppression paradigm						
Saline	7	2.3 ± 1.0	15.0 ± 5.9	5/7	7/7	7/7
LA	7	0	0	0	7/7 *	6/7 *
Late suppression paradigm						
Saline	7	3.3 ± 0.7	33.3 ± 7.0	6/7	7/7	7/7
LA	7	0	0	0	7/7	7/7

* One LA recipient did not have increased inflammatory cells compared with naïve in the right eye but did have injury in the left optic nerve, consistent with unilateral optic neuritis.

Table 2

Summary of morphological determination of tissue damage and immunofluorescence data from optic nerves.

	Early Suppression	Late Suppression
Toluidine Blue		
<u>Treatment</u>	<u>% Damage</u>	<u>% Damage</u>
Saline	14.91 ± 3.83	24.64 ± 3.54
LA	2.04 ± 0.35	8.41 ± 2.47
p value	0.001	0.004
Cross-sectional area of optic nerve		
<u>Treatment</u>	<u>µm²</u>	<u>µm²</u>
Saline	67520 ± 3897	64592 ± 2847
LA	64889 ± 3083	69242 ± 2336
p value	0.45	0.13
CD4 Immunofluorescence *		
<u>Treatment</u>	<u>% Area Stained</u>	<u>% Area Stained</u>
Saline	1.61 ± 0.22	2.22 ± 0.30
LA	0.59 ± 0.14	1.21 ± 0.27
p value	0.0019	0.0083
CD11b Immunofluorescence *		
<u>Treatment</u>	<u>% Area Stained</u>	<u>% Area Stained</u>
Saline	6.90 ± 0.99	8.62 ± 1.75
LA	2.41 ± 0.38	5.68 ± 0.81
p value	0.0004	0.395

* CD4 and CD11b % area stained of optic nerve sections from naïve mice (n= 4) was 0.004 ± 0.002 and 1.2 ± 0.18 respectively.