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Lamotrigine monotherapy does not provide protection against the loss of optic nerve axons in a rat model of ocular hypertension

Nephtali Marina [a,e], Marija Sajic [c], Natalie D. Bull [a,b], Alexander J. Hyatt [a,b], David Berry [d], Kenneth J. Smith [c], Keith R. Martin [a,b,f,*]

[a] Centre for Brain Repair, University of Cambridge, Cambridge, United Kingdom
[b] Department of Ophthalmology, University of Cambridge, Cambridge, United Kingdom
[c] Department of Neuroinflammation, UCL Institute of Neurology, London, United Kingdom
[d] Medical Toxicology Unit, Guy's and St. Thomas' Trust, London, United Kingdom
[e] UCL Department of Medicine, London, United Kingdom
[f] Cambridge NIHR Biomedical Research Centre, Cambridge, United Kingdom

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ABSTRACT

Sodium channel blocking agents such as lamotrigine are potent agents for neuroprotection in several animal models of neurodegenerative and neuroinflammatory disease. We therefore explored whether lamotrigine therapy was neuroprotective in a rat model of ocular hypertension characterized by axonal injury and selective loss of retinal ganglion cells. Twenty-seven male Wistar rats were injected subcutaneously twice daily with either lamotrigine (14 mg/kg/day) or vehicle. Two weeks after the first injection, experimental ocular hypertension was induced in one eye by 532 nm trabecular laser treatment. Intraocular pressure (IOP) was monitored by rebound tonometry and four weeks after the elevation of IOP the loss of optic nerve axons was quantified relative to eyes without either IOP elevation or lamotrigine exposure. In other animals with ocular hypertension, the optic nerves were examined by immunohistochemistry for the expression of the inducible form of nitric oxide synthase (iNOS) at 7 and 28 days. Four weeks after initiation of IOP elevation, no significant difference in axonal loss was observed between rats treated with lamotrigine (30.8% ± 10.5%) or vehicle (17.8% ± 5.7%) ($P = 0.19$, *T*-test). There was no significant difference in mean IOP, peak IOP and integral IOP exposure. Furthermore, optic nerve axon counts per unit integral IOP exposure were similar in both groups ($P = 0.44$). The optic nerves were not positive for the expression of iNOS. In conclusion, this study provides no evidence that lamotrigine is neuroprotective for RGC axons after four weeks of experimental ocular hypertension in the rat, in a model where axonal degeneration occurs in the absence of iNOS expression.

1. Introduction

Glaucoma is a neurodegenerative condition that is a leading cause of irreversible blindness worldwide (Quigley and Broman, 2006). Axonal injury at the level of the optic nerve head is a key event in the pathogenesis of glaucoma, and intraocular pressure is an important risk factor for the development and progression of the disease (Anderson, 1989). Pharmacological and surgical methods to lower IOP remain the first line treatments for the condition. However, visual loss continues at low IOP in a significant number of patients (Anderson, 2003). Therefore, understanding the pathophysiological mechanisms that lead to RGC degeneration is of vital importance to develop novel treatments to provide neuroprotection and, ultimately, to reduce its progression.

One mechanism that has been implicated in the development of axonal degeneration is the excessive accumulation of sodium ions within neurons and axons as a consequence of a dysfunction of the Na–K ATPase ('sodium pump') induced by a depletion of energy supply. Accumulation of intra-axonal sodium promotes the reverse action of the Na^+/Ca^{2+} exchanger, resulting in an influx of calcium ions and, thereby, calcium-mediated axonal degeneration (Bechtold and Smith, 2005; Stys, 2005). Using rodent models of multiple sclerosis, we and others have shown that axonal damage can be prevented using sodium channel blocking agents, such as

Abbreviations: IOP, Intraocular pressure; RGC, Retinal ganglion cell; iNOS, Inducible nitric oxide synthase; PBS, Phosphate buffered saline; PFA, Paraformaldehyde; I_{NaF}, Fast sodium current; I_{NaP}, Persistent sodium current.

* Corresponding author. Cambridge University Centre for Brain Repair, Forvie Site, Robinson Way, Cambridge CB2 0PY, United Kingdom. Tel.: +44 (0)1223 216427; fax: +44 (0)1223 331174.
E-mail address: krgm2@cam.ac.uk (K.R. Martin).

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flecainide, lamotrigine and phenytoin (Bechtold et al., 2004, 2006; Waxman, 2005, 2006). In the present study we explored whether lamotrigine can protect optic nerve axons against degeneration resulting from experimental ocular hypertension. Lamotrigine was chosen over other sodium channel blocking agents in anticipation of potential clinical use, because of its demonstrated effects in animal models of MS, and because it is well tolerated by patients (Kapoor, 2008; Mantegazza et al., 2010).

2. Methods

2.1. Experimental protocol

2.1.1. Drug administration

Animals (Wistar male rats, 200–300 g, $n = 27$) received lamotrigine (14 mg/kg/day, $n = 14$) or vehicle (50% dimethylsulphoxide in 0.1M phosphate buffered saline, pH 7.4, $n = 13$) by subcutaneous injection (0.1 ml), twice daily on a 12:12 h schedule. RGC neuroprotection therapy was established before superimposing the disease. In order to identify any direct effect of lamotrigine on IOP and to ensure lamotrigine was well tolerated by the animals, drug administration commenced 14 days before IOP elevation and was continued for 28 days after glaucoma induction. Lamotrigine solution was prepared fresh before each administration using commercially available tablets (200 mg; Approved Prescription Services Ltd., Leeds, UK) used in clinical practice. One tablet was finely crushed and dissolved in 10 ml of vehicle and the solution was filtered using a Millex GP Filter unit (0.22 μm, Millipore, Cork, Ireland) to prevent micro-organism inoculation, and to remove any suspended particulate matter. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines.

2.1.2. Experimental elevation of IOP

IOP elevation was achieved by external translimbal treatment to the aqueous outflow area with a 532-nm diode laser (Levkovitch-Verbin et al., 2002). Briefly, animals were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) and topical 1% tetracaine eyedrops administered. Laser energy was directed to the trabecular meshwork without the use of additional lenses. The laser beam was directed perpendicular to the trabeculum and parallel to the iris. Initial treatment was 60–70 spots of 50 μm size, 0.7 W power, and 0.6 s duration in the left eye only. Treatment was repeated at 1 week in all animals when the difference in IOP between the two eyes was less than 6 mm Hg (all animals in this study required re-treatment). IOP was measured under light general anaesthesia with a handheld tonometer (Tonolab; Tiolat, Finland). The average of three series of 6 IOP measurements was taken immediately before, and 1 day after each treatment and then weekly for the duration of the experiment. For each eye, multiple measures of IOP exposure were calculated including mean IOP, peak IOP, and positive integral IOP. Positive integral IOP was calculated as the area under the curve of IOP over time for the glaucomatous eye minus the area for the fellow control eye of each animal, during all periods when IOP was higher in the glaucomatous eye. This parameter captures the cumulative IOP exposure in glaucomatous eyes relative to fellow control eyes over the duration of the experiment in units of mm Hg-days (Martin et al., 2003). Mean IOP, peak IOP, and positive integral IOP were compared in different treatment groups by unpaired Student's *T*-tests.

2.2. Lamotrigine concentration

Four weeks after IOP elevation, animals were deeply anesthetized with an overdose of sodium pentobarbital, given 12 h after the last dose of lamotrigine had been administered, and a blood sample was obtained from the left ventricle. Samples were centrifuged at 5000 rpm for 5 min and the supernatant serum was collected for analysis. The concentration of lamotrigine was determined by a fully validated HPLC technique (Berry et al., 1992). Briefly, an internal standard was added to small (200 μl) serum samples and the pH was adjusted to 10.3 with TRIS buffer prior to extraction with solvent to remove the drug from the biomatrix. The solvent was evaporated to dryness and the residue was reconstituted in HPLC mobile phase prior to injection onto a reversed phase column with monitoring of the eluent by UV at 325 nm.

Quantification was by reference to calibrators that were spiked with lamotrigine across the range of values encountered during normal therapy. These calibrators were carried through the procedure with each batch of samples and every analytical run was subjected to internal quality control at three concentrations.

2.3. Preparation of optic nerves and semiquantitative grading of optic nerve damage

Optic nerve processing and axonal quantification were performed as described in detail previously (Bull et al., 2009; Marina et al., 2010). Briefly, four weeks after IOP elevation animals were killed by an overdose of pentobarbital and perfused transcardially with 0.1M phosphate buffered saline (PBS, pH 7.4, at room temperature) for two minutes at a rate of 50 mL/min, followed by 4% paraformaldehyde (PFA) in 0.1M PBS for five minutes. After perfusion, optic nerves were collected and stored in 4% PFA overnight at 4 °C and then transferred to 5% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4) for 48 h. Nerves were washed in 0.1M PB, pH 7.4 and postfixed in 1% osmium tetroxide for 3 h on a rotator. Nerves were washed, dehydrated in an ascending series of alcohols and embedded in resin. Semithin sections (1 μm) were collected and counterstained with 1% toluidine blue. A semiautomated method, adapted from Chauhan et al. (2006), was used to assess optic nerve axonal survival (see Marina et al., 2010 for full protocol). Optic nerve axonal survival was estimated by a masked observer. Briefly, bright field microscopy coupled to an image-analysis system with a digital camera and software (Nikon D300) was used to capture digital images of the whole optic nerve and total cross section area in pixels was estimated at low magnification (10×). Damage was visually inspected through the eyepieces at higher magnification (40×) to identify the zones where the damage was apparently uniform. The outer border of each damage zone was outlined and labelled on the digital image taken from the optic nerve cross section. The area of each damage zone was measured in pixels and the percentage of the nerve cross-area occupied by each zone was calculated. Individual axons were examined using a 100× objective under oil immersion, and one representative image composed of 92,000 pixels was taken from each damage area. The number of axons in each sample was counted using Image J 1.37c (National Institutes of Health, USA) and the particle analysis plug-ins bundle generated by the Wright Cell Imaging Facility Unit (WCIF, University Health Network Research, Canada). 16 bit images were enhanced using the shadows and thresholding functions to eliminate non-neural components. Images were then smoothed and axons were counted using the nucleus counter plug-in with the Otsu thresholding method. Total axon numbers in each damage zone were estimated and axonal counts in each nerve extrapolated. The weighted average equation was used to estimate the total number of axons in each optic nerve, based on percentage contribution of each damage zone to total optic nerve cross section area: