VINPOCETINE MODULATES METABOLIC ACTIVITY AND FUNCTION DURING RETINAL ISCHEMIA

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Abbreviations
LDH, lactate dehydrogenase; ERG, electroretinogram;

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Running title: Vinpocetine alters metabolism in retinal ischemia
Abstract

Vinpocetine protects against a range of degenerative conditions and insults of the central nervous system via multiple modes of action. Little is known however of its effects on metabolism. This may be highly relevant as vinpocetine is highly protective against ischemia, a process which inhibits normal metabolic function. This study characterizes vinpocetine’s effects on metabolism using the ischemic retina as a model. Vinpocetine reduced the metabolic demand of the retina following ex vivo hypoxia and ischemia to normal levels based on lactate dehydrogenase (LDH) activity. Vinpocetine delivered similar effects in an in vivo model of retinal ischemia-reperfusion, possibly through increasing glucose availability. Vinpocetine’s effects on glucose also appeared to improve glutamate homeostasis in ischemic Müller cells. Other actions of vinpocetine following ischemia/reperfusion, such as reduced cell death and improved retinal function, were possibly a combination of the drug’s actions on metabolism and other retinal pathways. Vinpocetine’s metabolic effects appeared independent of its other known actions in ischemia as it recovered retinal function in a separate metabolic model where the glutamate to glutamine metabolic pathway was inhibited in Müller cells. The results of this study indicate that vinpocetine mediates ischemic damage partly through altered metabolism and has potential beneficial effects as a treatment for ischemia of neuronal tissues.

KEY WORDS: vinpocetine, retina, ischemia, retinal metabolism, lactate dehydrogenase
1. Introduction

Vinpocetine, a herbal supplement extracted from the Vinca minor plant, acts beneficially in a range of degenerative metabolic conditions. In the brain, vinpocetine improves memory, cognitive function and protects against seizures and Alzheimer’s disease (9, 21, 27, 31, 78). In peripheral tissues, vinpocetine is linked to regulating blood circulation, preventing stroke, atherosclerosis and reducing inflammation (6, 10, 22, 28, 61, 62, 79). In the eye, vinpocetine provides neuroprotection against macular degeneration, diabetic retinopathy and visual impairment secondary to Meniere’s disease (4, 5, 37, 52, 63, 65).

Vinpocetine has consistently been shown to be protective in ischemic injury of many tissues including the brain, liver and retina (28, 31, 32, 44, 54, 74). Vinpocetine may protect against ischemia via multiple mechanisms. Vinpocetine inhibits Na⁺ channels and regulates cation entry into inner retinal neurons (44, 48, 56, 57), which prevents disruptions to ion homeostasis and glutamate excitotoxicity, events closely associated with the pathogenesis of ischemic damage (46). Vinpocetine may also prevent ischemic damage by scavenging of hydroxyl radicals and attenuating of oxidative stress (48, 49) or modifying immune responses by regulating microglial inflammation (77).

Vinpocetine may also protect against ischemia by reducing the metabolic demands of the insult. Indeed, vinpocetine increases glucose, ATP and phosphocreatine availability in the normal brain and cortical neurons, and astrocytes affected by hypoxia or ischemia (9, 25, 32, 40, 55, 62, 65). Metabolic actions are likely to be most significant in ischemia of the retina due to the high energy consumption of retinal neurons. However, the effects of vinpocetine on retinal metabolism are yet to be investigated.

This study investigates the metabolic actions of vinpocetine during retinal ischemia. We determine the effects of vinpocetine on metabolism by quantifying lactate dehydrogenase (LDH) activity, an enzyme that interconverts lactate and pyruvate and is a key indicator of retinal metabolic activity (1, 2, 50, 68, 69, 73). We assess vinpocetine’s actions using ex vivo and in vivo models of ischemia and show alterations in retinal metabolism with vinpocetine is associated with neuroprotection and improved retinal function post ischemic insult.

2. Material and methods

2.1 Animal ethics
All animal procedures were approved by the University of Auckland and University of New South Wales Animal Ethics committees and were in adherence with principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience.

2.2 Vinpocetine preparation

Vinpocetine (14-ethoxycarbonyl-(3α,16α-ethyl)-14,15-eburnamine) was obtained from Sigma-Aldrich (Auckland, New Zealand; Castle Hill, NSW, Australia) and prepared as a concentrated stock solution in dimethyl superoxide (DMSO). Vinpocetine was then diluted to 10 μM or 100 μM in saline or buffer so the final solution contained less than 0.1% DMSO. At this concentration, DMSO does not affect the activity of LDH, glucose or glutamate levels. Vinpocetine doses correspond to those shown to have a protective effect against damage in the brain and retina (9, 24, 44).

2.3 Ex vivo retinal culture models

*Ex vivo* experiments were performed as described in Nivison-Smith, et al. (44). Briefly, six week old adult Sprague Dawley rats were deeply anesthetized using domitor (5 mg/kg) and ketamine (60 mg/kg), euthanized and the eyes removed. A posterior eyecup was prepared by dissecting away the anterior eye structures and the eyecup mounted onto filter paper (pore size 0.8 μm; Gelman Sciences, MI, USA). The retinal pigmented epithelium, choroid and sclera were removed and isolated retinal samples placed in air tight incubation chambers.

Samples (n = 6 per treatment) subjected to normoxia were incubated at 37°C for 40 minutes with or without vinpocetine in Edwards buffer (125 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM dextrose, 2 mM CaCl₂, 1 mM MgCl₂) bubbled in 95% O₂/5% CO₂ (1, 23). These conditions closely mimic the *in vivo* retinal environment (67, 71). Hypoglycaemia was created by removing the glucose from the normoxia buffer and correcting the osmolarity with NaCl. Hypoxia was created by incubating retinae in normoxia buffer with 95% N₂/5% CO₂ (1, 44). Ischemia was created by incubating retinae in hypoglycaemic buffer in a nitrogen rich environment (95% N₂/5% CO₂).

2.4 *In vivo* ischemia-reperfusion model

Six week old Sprague Dawley rats (n = 24) were subjected to ischemia-reperfusion based on Sun et al. (60) model of elevating intraocular pressure. Rats were deeply anaesthetized and the corneal reflex inhibited topically with 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK). A 30 gauge needle was inserted 1mm anterior to the limbus and sterile saline (0.9% NaCl) pumped into the anterior chamber. Eye pressure was raised to 120 mmHg as measured with a sphygmomanometer for 1 h. The contralateral eye was used as a control and a needle was inserted
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into the eye but no saline was pumped in. Before, during and 1 hour after ischemia, ocular steroids/antibiotics (either Maxitrol (Alcon Ltd, New Zealand) or a combination of Flarex® (0.1% Fluorometholone; Alcon, Frenchs Forest, NSW, Australia) and antibiotics (Genoptic® 0.3%; Allergan Australia Pty Ltd, Gordon NSW, Australia) were applied topically. Rats were injected with antisedan (atipamezole) in the peritoneum and allowed to recover for 24 hours in the dark. Dark-adapted animals were then anaesthetized and given an intravitreal injection of saline or 100 µM vinpocetine into the ischaemic and contralateral control eye.

2.5 LDH activity assay

Retinal samples (n = 6) were homogenized in 0.9% NaCl and centrifuged at 5000g for 7 minutes. The supernatant was added to the LDH reagent containing 0.62 mM pyruvic acid and 0.23 mM NADH in 50 mM phosphate buffer (Trace, Noble Park, VIC, Australia) and LDH activity was determined as the change in NADH absorbance at 340 nm over the linear portion of the reaction for 5 replicates. Values were normalized against protein content of retinal samples assayed using the BCA protein assay (Pierce, Rockford, IL, USA). LDH released during cell death was determined by assaying LDH activity of an aliquot of incubation medium (1, 2, 69, 73). Negative controls were prepared with water instead of sample in the LDH reaction. In ex vivo models, LDH was measured immediately after incubation. For the in vivo model, LDH was measured 2 hours post intravitreal injection.

2.6 Glucose assay

Retinal samples were homogenized in 0.5 M perchloric acid with a glass-teflon homogenizer and periodically vortexing on ice before centrifuging at 5000g for 5 minutes. Homogenates were neutralized with 2 M KHCO₃ and the supernatant taken for glucose measurement using the Glucose HK assay kit per the manufacturer’s instructions (Sigma-Aldrich, Castle Hill, NSW, Australia). Glucose concentration was normalized against protein content in each sample assayed from the cell pellet using the BCA protein assay (Pierce, Rockford, IL, USA). Glucose was assayed at identical time points as LDH.

2.6 TdT-mediated dUTP nick end labeling (TUNEL) assay

Tissues from the in vivo ischemia-reperfusion model were collected approximately 2 hours post injection and fixed in 4% paraformaldehyde, 0.01% glutaraldehyde in phosphate buffer for 30 minutes. Samples were cryoprotected in sucrose and then sectioned in the vertical plane (14 µm thickness) and collected on pre-cleaned Superfrost plus slides (LabServ, New Zealand).
Cell death was determined using the TUNEL assay via the In Situ Cell Death Detection Kit (Roche Applied Science, Germany). Tissue sections were washed with phosphate buffered saline (PBS) then permeabilized with 0.1% Triton X-100, 0.1% tri-sodium citrate in 0.1 M PBS for 5 minutes on ice. Sections were incubated with the TUNEL reaction mixture (1:17 enzyme to nucleotide ratio) for 30 minutes at 37°C in a dark humid chamber. Negative control sections were incubated in reaction mixture without the TdT enzyme. Sections were rinsed several times and then mounted in Vectashield HardSet Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

Sections were visualized on a confocal microscope (Leica Microsystems Ltd., Heerbrugg) and a Leica DC 500 digital camera attachment (Twain model V4,1.0.0). All images were obtained using identical brightness and contrast settings. Quantification was performed in central retina ganglion cell layer as these cells are the most susceptible to cell death by ischemia (13, 18, 36). Data was expressed as the percentage of TUNEL positive cells among all DAPI labeled cells per mm linear area across at least 4 areas within central retina (n = 3).

### 2.7 Immunohistochemistry

Retinal sections were blocked with 6% goat serum, 1% bovine serum albumin, 0.1% (v/v) Triton-X for 1 h at room temperature then incubated overnight with primary antibodies: anti-goat Brn-3A (Santa-Cruz Biotechnology, SC-31984, 1:200) anti-mouse glial fibrillary acidic protein (Sigma Aldrich, G3893; 1:1000), anti-rabbit glutamate (Signature Immunologics, E100; 1:500), anti-rabbit glutamine (Abcam, ab9445; 1:500), anti-mouse glutamine synthetase (Millipore, MAB302; 1:1000). Primary antibodies were detected with AlexaFluor 488 or AlexaFluor 594 conjugated secondary antibodies diluted 1:500 (Life Technologies, Mulgrave, VIC, Australia). Sections were counterstained with DAPI and visualized on a confocal microscope as described for TUNEL labelling. Staining for each marker was performed in a single experimental run and all images were obtained under identical brightness and contrast settings so inter-sample comparisons could be made. Representative images in Figure 5 were adjusted for brightness and contrast in Adobe Photoshop (version 8, Mountain View, CA).

### 2.8 Image analysis

Glial fibrillary acidic protein (GFAP) immunoreactivity was quantified by thresholding labelled images to remove background fluorescence (all pixels were valued between 0-255 and those under 20 removed) and converting to binary using Image J (National Institutes of Health). The nerve fiber layer (NFL)/ganglion cell layer (GCL) and inner plexiform layer (IPL) was delineated based on DAPI labelling.
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using the polygon tool in ImageJ and GFAP was expressed as the percentage of GFAP positive pixels within the total area of the specific retinal layer using the measure tool in ImageJ.

Glutamine synthetase (GS) labelled images were converted to greyscale and the total number of GS positive pixels across the total retinal area quantified. The GS pixel value was quantified within the NFL/GCL using the measure tool in ImageJ.

Glutaminate immunoreactivity was quantified as described in Figure 1. Retinal samples were co-labelled with DAPI, glutamate and GS and imaged on a confocal microscope as a five, 0.9µm stack. The final image for quantification was composed of the pixels of maximum value amongst the 5 images (Figure 1, step 1). The image was separated into individual channels (Figure 1, step 2) and the GS images thresholded and converted to binary (Figure 1, step 3). The threshold limits were set so that most GS processes were represented in the final binary image and only a small proportion were lost based on similarity to noise intensities. The glutamate immunoreactivity of Müller cells was determined by subtracting the binary GS images from glutamate images using the Image calculator tool in ImageJ so only glutamate immunoreactivity co-localized within GS labelling was visible (Figure 1, step 4). The reverse subtraction was performed to give glutamate immunoreactivity in non-Müller cell space (i.e. neurons, other glia). Glutamate immunoreactivity of all cells in the sample was based on the unmodified glutamate image. Glutamate images were segmented into the IPL, and GCL/NFL based on DAPI labelled images. Glutamate within a region of interest was measured as mean pixel value (ranging 0-255) per µm² area analyzed using the measure tool in ImageJ. For each retinal area, 5 retinae were assessed and a total area of at least 50,000 µm² was assessed per retinae.

Immunoreactivity was then normalized to the glutamate content of the control eye treated with saline. Glutamine immunoreactivity was determined in an identical manner. For glutamate:glutamine ratios, the pixel values were normalised to total GS pixels in each sample and the mean of each treatment group compared for the final ratio. All images used for comparative analysis were stained, imaged and quantified under identical conditions in a single experimental run so estimation of the net pixel value across samples could be performed.

2.9 Electoretinograms (ERG)

ERGs were used to determine the functional activity of specific retinal neurons including photoreceptors which primarily contribute to the early negative a-wave, ON bipolar cells which contribute to the subsequent, positive b-wave and amacrine cells which contribute to small oscillating wavelets between the a- and b-wave called oscillatory potentials (Hood & Birch, 1990).
ERGs were conducted 1 hour after injection of saline or vinpocetine (25 hours post ischemia) as described previously (16, 45, 58). Under dim red light illumination, pupils were dilated with 0.5% tropicamide (Bausch & Lomb, North Ryde, NSW, Australia) and Ag/AgCl contact electrodes were placed on each cornea. A reference electrode was placed over the lower incisors in contact with the tongue and a ground electrode in the tail (Grass Technologies, West Warwick, RI). Rats were exposed to a single 1 ms light flashes of increasing intensity (-3.9 - 2.1 log cd.s/m²) from a Ganzfeld integrating sphere (Photometric Solutions International, Huntingdale, VIC, Australia). Responses were recorded as the average of three flashes at each intensity at 4 kHz with band pass settings of −3 dB at 0.3 to 1000 Hz (ML785, Powerlab/BSP, 1000× amplification; AD Instruments).

ERG data was plotted and analyzed using Michaelis-Menten function of the amplitude-intensity relationship of mixed cone and rod response (41). The a-wave amplitude was measured as the minimum value of the ERG waveform trough and the amplitude of the b-wave was measured as the value from trough to the peak of the waveform (58, 66). The oscillatory potential amplitude was measured as the peak of the largest wavelet between the a-wave and b-wave peak.

**2.10 Metabolic inhibition assay**

Pharmacological inhibition of retinal metabolism was performed based on Bui et al (15). Normal, dark-adapted, six week old Sprague Dawley rats (n = 5) were deeply anaesthetized and L-Methionine sulfoximine (Sigma Aldrich, Castle Hill, NSW, Australia) diluted in sterile saline was injected intravitreally into each eye to a final concentration of 23 mM. ERG recordings were taken at a single light intensity (2.1 log cd.s/m²) 40 minutes post injection. At 90 minutes post injection, 100 µM vinpocetine was intravitreally injected into the left eye and an equal volume of saline into the right eye. A second ERG recording was taken 70 minutes post Vinpocetine treatment. ERG a- and b-wave peaks were plotted relative to baseline values.

**2.11 Statistical analysis**

Statistical analysis was performed using two-way ANOVA to compare the effect of drug concentration on LDH activity and experimental condition. If statistical significance was reached, post hoc Tukey’s analysis was conducted. Student’s t-test was used for paired comparisons (control vs treated). All data are presented as mean ± standard error of the mean (SEM). All data was confirmed for normality using the Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lillie for an alpha value of 0.05.

**3. Results**
3.1 The metabolic effects of vinpocetine on following metabolic insult

Metabolism was assessed as changes in metabolic rate (based on LDH activity) and cell death (based on release of LDH into the medium). We found normal LDH activity of the retina was 7.1 ± 0.2 U/mg protein (Fig. 2A). Comparatively, hypoxia, hypoglycaemia and ischemia (Fig. 2B-D, black columns) significantly increased LDH activity in the retina (Student’s t-test, p < 0.05 for all insults). These insults also significantly increased LDH release into the incubation medium (Student’s t-test, p < 0.001 for all insults) compared to normoxia.

The use of 10 µM and 100 µM vinpocetine significantly increased LDH activity in the normal retina by 24% and 14%, respectively (two-way ANOVA, post-hoc analysis, p < 0.05; Fig. 2A). Vinpocetine had no effect on LDH release of the normal retina into the medium (two-way ANOVA, p = 0.6). In hypoxia, both dosages of vinpocetine further increased LDH activity from 11% to 38% (two-way ANOVA, post-hoc analysis, 10 µM: p < 0.05, 100 µM: p < 0.001). In contrast, vinpocetine reduced cell death in hypoxia, lowering of LDH release to levels of the normal retina (p < 0.001). In hypoglycaemia, vinpocetine did not significantly alter the elevated LDH activity or LDH release into the medium (two-way ANOVA, retina: p = 0.5, medium: p = 0.1; Fig. 2C). In ischemia, vinpocetine significantly reduced LDH activity to baseline at both dosages (two-way ANOVA, post-hoc analysis, p < 0.05). Vinpocetine, however had no effect on ischemic cell death as LDH release in ischemic medium remained elevated with 10 µM and 100 µM vinpocetine (two-way ANOVA, p = 0.5).

3.2 The effect of vinpocetine on retinal metabolism in vivo

Ex vivo incubations indicated that ischemia was the only insult in which vinpocetine reduced metabolic activity to normal levels. We further investigate these changes using an in vivo ischemia-reperfusion model to identify possible mechanisms for vinpocetine’s actions (14, 58). In the in vivo model, one eye was subjected to ischemia followed by 24 hour reperfusion. Contralateral eyes were used as controls. Following reperfusion, an intravitreal injection of saline or 100 µM vinpocetine was administered. Contralateral eyes received identical injection volumes.

LDH activity in contralateral control eyes were comparable to the ex vivo normoxia model (Student’s t-test, p = 0.5; Fig. 3A). The LDH activities of ischemia-reperfusion eyes were significantly higher than control eyes (Student’s t-test, p < 0.05). Vinpocetine had no effect on control eyes but significantly reduced LDH activity of ischemia-reperfusion eyes to normal levels (Student’s t-test, p < 0.001; Fig 3A).
We hypothesised that vinpocetine’s effects on retinal metabolism may involve glucose as vinpocetine increases glucose levels in ischaemic brain tissue (28, 55, 62). We found no difference in glucose concentration between control and ischemic-reperfusion eyes treated with saline (Student’s t-test, p = 0.2; Fig. 3B). Vinpocetine significantly increased glucose concentration in control and ischemic eyes (Student’s t-test, control: p < 0.05, ischemia: p < 0.01). Elevated glucose was also observed with vinpocetine treatment of the ex vivo normoxia (Student’s t-test, p < 0.01, data not shown). Other metabolites such as ATP and phosphocreatine were unaltered in the normal retina following vinpocetine treatment (data not shown).

Finally, the in vivo ischemia-reperfusion model showed significant cell death in the ganglion cell layer suggesting loss of ganglion cells and/or displaced amacrine cells, glial and endothelial cells (Fig. 3C-D). Vinpocetine treatment significantly decreased cell death in the ganglion cell layer 2 hours post drug administration (Student’s t-test, p < 0.05). This contrasted the ex vivo model and was possibly due to differences in the experimental timelines of vinpocetine administration in each model. Few TUNEL positive cells were present in the ganglion cell layer of contralateral control eyes with and without vinpocetine treatment (data not shown).

### 3.3 The effect of vinpocetine on Müller cells

We suspected vinpocetine may act on Müller cells as these are the primary cells involved in retinal metabolism (12, 35, 50, 64). We assessed the reactivity of Müller cells via the glial stress marker, glial fibrillary acidic protein (GFAP; 12). GFAP was mostly restricted to the nerve fibre layer (NFL) and ganglion cell layer (GCL) of control eyes treated with saline or vinpocetine (Fig. 4A-C). GFAP was significantly elevated in ischemia-reperfusion eyes with immunoreactivity extending from NFL to the inner plexiform layer (Student’s t-test, p < 0.001). Vinpocetine reduced GFAP immunoreactivity in ischemia-reperfusion eyes but these levels were still significantly greater than control eyes (Student’s t-test, GCL: p < 0.001, IPL: p < 0.05). Müller cells were also assessed for the established Müller cell marker, glutamine synthetase (GS). In all treatments, the total number of GS pixels remained constant suggesting the total area occupied by Müller cells did not change with ischemic insult or vinpocetine treatment (data not shown). However, the intensity of GS pixels was significantly decreased following ischemia-reperfusion indicating the level of enzyme was reduced (Student’s t-test, p < 0.05; Fig. 4D). Vinpocetine had no effect on GS pixel value in control or ischemia-reperfusion eyes (Student’s t-test, control: p = 1.0, ischemia: p = 0.9).

We further investigated the effects of vinpocetine on Müller cells by assessing changes to a key biochemical pathway of the cell, the conversion of glutamate to glutamine (11). This pathway is
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closely linked to energy metabolism (reviewed in 33) and thus vinpocetine’s actions on glucose availability could alter glutamate metabolism in Müller cells. Additionally, changes in Müller cell glutamate may affect neurons as retinal neurotransmitters are sourced from Müller cells. Semi-quantitative analysis of glutamate immunoreactivity (Figs. 5-6A-F) showed a significant increase in glutamate in the inner plexiform layer (IPL) following ischemia-reperfusion (Student’s t-test, p < 0.05; Fig. 6A). Vinpocetine reduced glutamate levels of the IPL to that of control eyes (Student’s t-test, p = 0.6). No other retinal layers showed significant changes in glutamate immunoreactivity following ischemia-reperfusion (Fig. 6B, data not shown for other layers).

To assess changes specifically occurring within Müller cells, we segmented the retinal space into Müller cells and non-Müller cells based on positive and negative GS immunoreactivity (Fig. 1). Glutamate immunoreactivity was significantly elevated in the Müller cell processes in the IPL (Student’s t-test, p < 0.01, Fig. 6C) and slightly elevated in the endfeet (p = 0.1; Fig. 6D) following ischemia-reperfusion. Vinpocetine reduced glutamate immunoreactivity of ischemic Müller cell processes in the IPL to levels comparable to control eyes (Student’s t-test, processes: p = 0.6).

Glutamate metabolism was not altered in non-Müller cells of the GCL/NFL in ischemic eyes (Student’s t-test, p = 0.7) or with vinpocetine treatment (Student’s t-test, p =0.7; Fig. 6E). However, visual inspection of immunolabelled tissue indicated highly glutamate immunoreactive cell somata within the GCL (Fig. 5). As non-Müller cell space could be occupied by ganglion cells, displaced amacrine cells and glia, we isolated the effects of vinpocetine on ganglion cells alone by co-localizing glutamate immunoreactivity with the ganglion cell marker, Brn-3A (Fig. 6F). Ischemia-reperfusion significantly increased glutamate content of ganglion cells (Student’s t-test, p < 0.001) but unlike Müller cells, vinpocetine had no effect on glutamate content of ischemic ganglion cells (p = 0.8).

Ischemia-reperfusion also significantly increased glutamine immunoreactivity of Müller cell endfeet (Student’s t-test; p <0.01; Fig. 6G). Vinpocetine altered glutamine immunoreactivity in both control and ischemic eyes indicating the drug affects multiple amino acids within the Müller cell (Fig. 6H). Comparison of the glutamate:glutamine ratio within Müller cell endfeet showed a 0.4 fold increase following ischemia. Vinpocetine reduced this ratio to levels below baseline. Very little glutamate immunoreactivity was found in Müller cell somata under all conditions (data not shown).

3.5 The effect of vinpocetine on retinal function during ischemia
ERGs were performed in the *in vivo* ischemia-reperfusion rat to determine if vinpocetine’s actions on retinal metabolism had functional implications (Fig. 7). ERGs indicate retinal function by measuring the electrical responses of retinal cells to light stimuli of specific intensity and duration (30). Control eyes showed a typical normal ERG waveform with an initial, negative a-wave due to the response of photoreceptors and the subsequent, positive b-wave due to the response of ON bipolar cells (Fig. 7A,C-D). Vinpocetine had no effect on the a- and b-wave responses of control eyes. However, in ischemia-reperfusion, the a- and b-wave responses were significantly reduced indicating decreased photoreceptor and inner retinal neuron function (Fig. 7B-D). Vinpocetine treatment significantly improved the b-wave (inner retina) response of ischemic eyes to levels comparable to control eyes (two-way ANOVA, p<0.001). Vinpocetine however did not change a-wave (photoreceptor) response as this remained significantly lower in ischemic eyes than control eyes. The oscillatory potentials which indicate amacrine cell function were not altered under any treatments (Fig. 7E).

### 3.6 The metabolic effects of vinpocetine occur in other models of metabolic insult

Previous studies indicate vinpocetine has multiple actions during ischemia including regulation of cation entry through ionotropic glutamate receptors (44). Ionotropic glutamate receptors have significant ATP requirements and thus may be the cause of vinpocetine’s metabolic actions (3). To determine if vinpocetine’s metabolic effects were independent or a secondary effect of its other actions in ischemia, the metabolic effects of vinpocetine were tested in an *in vivo* model that directly blocks a metabolic pathway in Müller cells. Normal retinae were treated with L-Methionine sulfoximine (MSO) which inhibits GS and impairs glutamate to glutamine conversion resulting in a reduction of the b-wave amplitude over a period of 90 minutes (7, 15, 51). Impaired Müller cell metabolism was evident as a decrease in b-wave amplitude on ERG but no change in a-wave amplitude after 40 minutes (Fig. 8). After 90 minutes, MSO inhibited retinae were treated with saline or vinpocetine for a further 70 minutes. There was continued decay of the b-wave for the saline treated retina whilst vinpocetine prevented further b-wave loss leading to a significant enhanced response (Fig. 8C: Student’s t-test, p < 0.05). Such an effect supports a metabolic drug action within the inner retina. Vinpocetine also decreased a-wave amplitude suggesting the effect of MSO extended beyond Müller cells to photoreceptors (Fig. 8B).

### 4. Discussion

We found that vinpocetine can modulate retinal metabolic activity and function in normal, *ex vivo* and *in vivo* models of ischemia when measured via LDH activity. Vinpocetine reduced the LDH activity of ischemic retinae to normal levels, and increased glucose availability. Vinpocetine’s actions on glucose also likely explained its ability to modify glutamate homeostasis in ischemic Müller cells.
Other actions of vinpocetine post ischemia included reduced ganglion cell death and improved inner retinal function. Although these latter effects could involve vinpocetine’s other modes of action, we confirmed that vinpocetine’s metabolic actions could be observed in models independent of ischemia. These metabolic actions overlap those observed for vinpocetine in brain tissue suggesting the drug may be effective in the treatment of ischemia in retina and other parts of the central nervous system.

4.1 Vinpocetine enhances normal retinal metabolism
Vinpocetine increased LDH activity of the normal retina likely through increased glucose availability. Multiple studies confirm that retinal glucose increases LDH activity (47, 50, 68, 70). Vinpocetine also facilitates glucose uptake and release in the brain suggesting the drug modulates metabolic activity in neural systems via glucose (28, 55, 61, 62). Vinpocetine may also act directly on LDH by accelerating the reverse reaction of lactate to pyruvate as it lowers lactate levels in the rat cortex (2, 32). This effect was only seen in the ex vivo model where LDH activity was measured immediately after vinpocetine treatment and not the in vivo model where LDH activity was measured 2 h after vinpocetine administration. This short-lived effect reinforces the concept that vinpocetine regulates glucose uptake and release kinetics rather than having a more permanent effect on glucose metabolism (29, 61).

4.2 Vinpocetine modulates retinal metabolism following ischemia
Both ex vivo and in vivo ischemia models showed elevated retinal LDH activity following insult. Elevated LDH activity is associated with other models of retinal damage and changes in ATP demand confirming it as an indicator of retinal stress (1, 2, 8, 68, 69, 73, 75). Vinpocetine reduced LDH activity in the ischemic retina despite also increasing glucose levels. Vinpocetine acts similarly in the brain, reducing LDH activity in damaged brain tissue (21) despite improving oxygen and glucose levels (28, 55, 62). This suggests vinpocetine may lower metabolic demand in ischemia via mechanisms other than modulating glucose metabolism. Vinpocetine may increase the availability of other metabolites as its actions in the MSO inhibited retina was similar to those observed by Bui et al. (15) when lactate, succinate, pyruvate or α-ketoglutarate was supplied to the MSO inhibited retina. Vinpocetine’s metabolic actions may also be secondary to its other known actions on glutamate receptors in ischemia (44) however results in our other model for metabolic insult suggest the drug’s metabolic actions are primary.

4.3 Vinpocetine modifies Müller cell function following ischemia
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Vinpocetine’s metabolic actions likely involve Müller cells as these are the primary cells involved in retinal metabolism (12, 35, 50, 64). Indeed, we found vinpocetine slightly lowered GFAP immunoreactivity in ischemia-reperfusion suggesting the drug possibly protects against retinal stress. Vinpocetine also modulated Müller cell amino acid homeostasis. Altered amino acid metabolism is a hallmark of retinal ischemia and other retinal pathologies (19, 20, 26, 33, 42, 43, 60). In this study, Müller cell glutamate:glutamine ratios were elevated following ischemia-reperfusion. Vinpocetine lowered Müller cell glutamate:glutamine ratios to normal levels which may explain its ability to improve retinal function in ischemic eyes. Vinpocetine had no effect on GS expression suggesting the drug may not regulate amino acid homeostasis by directly acting on this enzyme in the glutamine/glutamate conversion pathway. Vinpocetine’s actions on glucose however may explain changes in Müller cell glutamate levels as Napper and Kalloniatis (42) found internal stores of glucose could reduce glutamate accumulation of post-mortem ischemic Müller cells.

4.4 Vinpocetine improves retinal function post ischemia

We showed for the first time that vinpocetine improves retinal function following ischemia-reperfusion in an ERG. These functional changes are likely associated with vinpocetine’s actions on glucose which improves glutamate homeostasis in Müller cells and prevents neuron dysfunction from disrupted neurotransmitter availability. Other ERG studies have shown improved retinal function secondary to glucose administration (17, 34, 43).

The rat middle retinal layers display low oxygen levels (72) and the known high levels of LDH activity in these layers (38) strongly suggest that anaerobic metabolism is the major energy source for bipolar cells and other retinal neurons located in the mid-retina. Vinpocetine returned the b-wave (bipolar cell response) to baseline levels following ischemia but did not recover the a-wave (photoreceptor response). This may be linked to vinpocetine’s other actions as a phosphodiesterase 1 (PDE1) inhibitor as others have shown vinpocetine can also partly inhibit PDE6, a key enzyme in the photoreceptor phototransduction pathway (76). This and the likely metabolic pathways, also possibly explains the recovery of the b-wave by vinpocetine in the in the MSO-treated retina with a concurrent decrease in the a-wave. Decreases in a-wave responses have also been shown to be transient for following treatment with PDE5 inhibitors (45). Vinpocetine’s effect on the a-wave is also likely to be transient as no anatomical damage such as photoreceptor death was observed. As ERGs are more sensitive techniques to detect ischemia damage than morphological evidence (53), future studies with longer observational time frames may be needed to quantify the full extent of vinpocetine’s actions on retinal metabolism during ischemia.
5. Conclusion

This study found that vinpocetine alters retinal metabolic activity under normal, hypoxic and ischemic conditions. In ex vivo and in vivo models of ischemia, vinpocetine decreased LDH activity and these actions were associated with increased glucose availability. Vinpocetine’s metabolic actions also improved glutamate metabolism in ischemic Müller cells despite changes in GS levels. Vinpocetine prevented cell death and significantly improved retinal function of inner retinal neurons post ischemia likely through its actions on retinal metabolism combined with actions on glutamate receptor activation. These results indicate vinpocetine has potential functional and neuroprotective applications in the treatment of ischemia in the retina and possibly other CNS tissues.

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Figure legends

Figure 1: Image processing for glutamate immunoreactivity measurements. Retinal samples labelled with DAPI, glutamate and glutamine synthetase (GS) were imaged as in step 1 then separated into individual red, green and blue channels in step 2. GS labelled images were thresholded to remove background fluorescence and the remaining GS immunoreactive pixels converted to a binary image (step 3). DAPI images were used to identify retinal layers. The original glutamate image was either left unmodified to assess glutamate content in all retinal cells or subtracted from the binary GS image from step 3 to give glutamate immunoreactivity within GS pixels for glutamate content of Müller cells (step 4). Images generated from the reverse subtraction (i.e. glutamate not co-localized with GS) were used to quantify the glutamate content of non-Müller cells (last image, step 4). Images in step 4 were separated into the IPL and the GCL/NFL using the DAPI image from step 3 before quantification. Abbreviations: IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. Scale bar is 20 µm.

Figure 2. The effect of vinpocetine on retinal LDH activity during (A) normoxia, (B) hypoxia, (C) hypoglycaemia and (D) ischemia. LDH activity in the tissue is shown on the left side of the graph and LDH activity leaked into the medium is shown on the right. Data represents the mean ± SEM of six retinae per condition. The dotted line in B-D represents the values found in normoxia with no additive. Statistical differences between treatment groups (black columns) and normoxia (the dotted line) are indicated by hash (#) symbols using a Student’s t-test. Statistical differences between vinpocetine groups (white, grey columns) and the no additive group (black columns) are shown with asterisks (*) based on a two-way ANOVA, (p < 0.05) followed by post hoc comparison (Tukey’s test). Abbreviations/symbols: vinp, vinpocetine, # = p < 0.05, * = p < 0.05, ### = p < 0.001, *** = p < 0.001.

Figure 3. The effect of vinpocetine on metabolic activity and cell death in an in vivo ischemia-reperfusion animal model. (A) LDH activity and (B) glucose concentration of control and ischemic retinae following an intravitreal injection with saline or vinpocetine. The value of control, saline injected eyes (first column) was continued as a dotted line for easy comparison between treatments. Cell death was assessed with (C) TUNEL labelling and (D) cell counts of TUNEL positive cells in the ischemia-reperfusion retinae after saline or vinpocetine treatment. Data is presented as the percentage of TUNEL positive cells among DAPI labelled cells in the GCL. Statistical differences between treatment groups and control (first black column and the dotted line) are indicated by hash (#) symbols based on Student’s t-test. Symbols: "" = p < 0.01. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer. All other abbreviations and symbols as in Figures 1-2. Scale bar in (C) is 50 µm.
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Figure 4. The effect of vinpocetine on Müller cell protein expression in an in vivo ischemia-reperfusion animal model. (A) Representative images of glial fibrillary acidic protein (GFAP) labelling and quantification of GFAP immunoreactivity in the (B) GCL/NFL and (C) IPL of control and ischemic retinae following saline or vinpocetine treatment. GFAP immunoreactivity is presented as the percentage area of GFAP pixels within the total retinal area specified. (D) Quantification of GS based on mean pixel value per µm² linear area within the GCL/NFL. Hash (#) symbols indicate significant differences between a treatment and the control, saline injected retina (first column and dotted line). Abbreviations/symbols: GS, glutamine synthetase, GFAP, glial fibrillary acidic protein. All other abbreviations and symbols as in Figures 1-2. Scale bar in (A) is 50 µm.

Figure 5. Representative images of glutamate content in the retina following vinpocetine treatment. Control eyes treated with (A-C) saline or (D-F) vinpocetine and ischemic eyes treated with (G-I) saline or (J-L) vinpocetine were labelled with glutamate (Glu; A, D, G, J), glutamine synthetase (GS; B, E, H, K) and co-localized (C, F, I, L). DAPI labelling has been omitted for clarity. Abbreviations: Glu, glutamate. Abbreviations as in Figures 1-2. Scale bar is 20 µm.

Figure 6. Quantification of relative glutamate and glutamine immunoreactivity following vinpocetine treatment. Glutamate content was determined in (A) the IPL and (B) ganglion cell/nerve fiber layer (GCL/NFL). Glutamate immunoreactivity co-localized with glutamine synthetase (GS) was then used to determine the glutamate content of (C) Müller cell inner retinal processes in the IPL and (D) Müller cell endfeet in the GCL/NFL. Glutamate pixels not co-localized with GS were used to determine the glutamate content in (E) non-Müller cells of the GCL/NFL which included ganglion cells, displaced amacrine cells astrocytes and microglia. (F) Glutamate content of ganglion cells alone was determined by co-localizing glutamate with Brn-3A. Glutamine immunoreactivity co-localized with GS was also used to determine (G) the glutamine content of Müller cell endfeet and (H) the ratio of mean glutamate:glutamine levels from (D) and (G) were compared. Immunoreactivity was expressed relative to the control eye treated with saline in each condition. The dotted line indicates a relative value of 1 (baseline value) for easy comparison between data columns. All samples were stained and imaged under identical conditions in a single experimental run so relative glutamate immunoreactivity could be determined between samples. Hash (#) symbols indicate any significant difference between treatment groups and the control, saline injected retina (first column/dotted line). Abbreviations/symbols as in Figures 1-3.
Figure 7. The effect of vinpocetine on retinal function in an in vivo ischemia-reperfusion animal model. Representative raw ERG waveforms in (A) control and (B) ischemic animals, 1 hour post injection with saline (black line) or vinpocetine (grey line). (C) The a-wave (photoreceptor) responses, (D) b-wave (ON bipolar cells) responses and (E) oscillatory potentials (amacrine cell; OP) responses of control and ischemic animal following vinpocetine treatment. The hash symbols (#) indicate significant differences between any treatment and the control, saline injected retina (open circles). Symbols as in Figures 2-3.

Figure 8. The effect of vinpocetine in an in vivo model of metabolic inhibition using MSO. (A) Representative raw ERG waveforms of retinae at baseline (i.e. before MSO inhibition; dotted line) and MSO inhibited retinae which had been inhibited with MSO for 90 minutes then treated with saline (grey line) or vinpocetine (black line) for a further 70 minutes (i.e. 160 minutes post baseline). The mean ± SEM amplitude of the (B) a-wave and (C) b-wave was determined before MSO treatment (baseline), 40 minutes after MSO administration (MSO inhibition) and 70 minutes after vinpocetine or saline treatment (treated). A complete experimental timeline including timepoints for ERG recordings is shown below panel (C). All symbols and abbreviations as in Figure 1-3.
A. normoxia

B. hypoxia

C. hypoglycaemia

D. ischemia

Legend:
- **no additive**
- **10μM vinpocetine**
- **100μM vinpocetine**