Vinpoce-tine modulates metabolic activity and function during retinal ischemia

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Submitted 26 August 2014; accepted in final form 17 February 2015

Vinpocetine modulates metabolic activity and function during retinal ischemia. Am J Physiol Cell Physiol 308: C737–C749, 2015. First published February 18, 2015; doi:10.1152/ajpcell.00291.2014.—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 that inhibits normal metabolic function. This study uses the ischemic retina as a model to characterize vinpocetine’s effects on metabolism. Vinpocetine reduced the metabolic demand of the retina following ex vivo hypoxia and ischemia to normal levels based on lactate dehydrogenase 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.

vinpocetine; retina; ischemia; retinal metabolism; lactate dehydrogenase

Vinpocetine modulates metabolic activity and function during retinal ischemia. Am J Physiol Cell Physiol 308: C737–C749, 2015. First published February 18, 2015; doi:10.1152/ajpcell.00291.2014.—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 that inhibits normal metabolic function. This study uses the ischemic retina as a model to characterize vinpocetine’s effects on metabolism. Vinpocetine reduced the metabolic demand of the retina following ex vivo hypoxia and ischemia to normal levels based on lactate dehydrogenase 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.

Vinpocetine, an herbal supplement extracted from the Vinca minor plant, acts beneficially in a range of degenerative metabolic conditions. In the brain, vinpocetine improves memory and cognitive function and protects against seizures and Alzheimer’s disease (9, 21, 27, 31, 78). In peripheral tissues, vinpocetine is linked to regulation of blood circulation, prevention of stroke and atherosclerosis, and reduction of 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 hydroxyl radicals and attenuating oxidative stress (48, 49) or by 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 because of 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 activity of lactate dehydrogenase (LDH), an enzyme that interconverts lactate and pyruvate and is a key indicator of retinal metabolic activity (1, 2, 50, 68, 69, 73). Using ex vivo and in vivo models of ischemia, we assess vinpocetine’s actions and show that alterations in retinal metabolism with vinpocetine are associated with neuroprotection and improved retinal function after ischemic insult.

MATERIALS AND METHODS

Ethical treatment of animals. All animal procedures were approved by the University of Auckland or the 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.

Vinpocetine preparation. Vinpocetine [14-ethoxyxcarbonyl-(3a,16x-ethyl)-14,15-eburnamime] was obtained from Sigma-Aldrich (Auckland, New Zealand; Castle Hill, NSW, Australia) and prepared as a concentrated stock solution in DMSO. Vinpocetine was then diluted to 10 or 100 μM in saline or buffer, so the final solution contained <0.1% DMSO. At this concentration, DMSO does not affect LDH activity or 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).

Ex vivo retinal culture models. Ex vivo experiments were performed as described by Nivison-Smith et al. (44). Briefly, 6-week-old adult Sprague-Dawley rats were deeply anesthetized using medetomidine (Domitor, 5 mg/kg) and ketamine (60 mg/kg) and euthanized, and the eyes were removed. The anterior eye structures were dissected away to prepare a posterior eyecup, which was mounted onto filter paper (0.8-μm pore size; Gelman Sciences). The retinal pigmented epithelium, choroid, and sclera were removed, and isolated retinal samples were placed in air-tight incubation chambers.

Samples (n = 6 per treatment) subjected to normoxia were incubated at 37°C for 40 min 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, and 1 mM MgCl2) bubbled

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in 95% O₂-5% CO₂ (1, 23). These conditions closely mimic the in vivo retinal environment (67, 71). Hypoglycemia was achieved by removal of glucose from the normoxia buffer and correction of osmolarity with NaCl. Hypoxia was achieved by incubation of retinae in normoxia buffer with 95% N₂-5% CO₂ (1, 44). Ischemia was achieved by incubation of retinae in hypoglycemic buffer in a nitrogen-rich (95% N₂-5% CO₂) environment.

In vivo ischemia-reperfusion model. Six-week-old Sprague-Dawley rats (n = 24) were subjected to ischemia-reperfusion based on the model of intraocular pressure elevation described by Sun et al. (60). Rats were deeply anesthetized, and the corneal reflex was inhibited by topical application of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK). A 30-gauge needle was inserted 1 mm anterior to the limbus, and sterile saline (0.9% NaCl) was pumped into the anterior chamber. Eye pressure was raised to 120 mmHg, as measured with a sphygmanometer, for 1 h. The contralateral eye was used as a control: a needle was inserted into the eye, but no saline was pumped in. Before, during, and 1 h after ischemia, ocular steroids/antibiotics [neomycin, polymyxin B, and dexamethasone (Maxitrol, Alcon) or a combination of 0.1% fluorometholone (Flarex, Alcon, Frenchs Forest, NSW, Australia) and antibiotics (gentamicin sulfate, Genoptic 0.3%, Allergan Australia), and antibiotics (gentamicin sulfate, Genoptic 0.3%, Allergan Australia, Gordon NSW, Australia)] were applied topically. Antisedan (atipamezole) was injected into the peritoneum, and the rats were allowed to recover for 24 h in darkness. Dark-adapted animals were anesthetized, and saline or 100 µM vinpocetine was injected intravitreally into the ischemic and the contralateral control eye.

LDH activity assay. Retinal samples (n = 6) were homogenized in 0.9% NaCl and centrifuged at 5,000 g for 7 min. 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 five replicates. Values were normalized against protein content of retinal samples assayed using the bichinonic acid protein assay (Pierce, Rockford, IL). LDH released during cell death was determined by assay of 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. In the in vivo model, LDH was measured 2 h after intravitreal injection.

Glucose assay. Retinal samples were homogenized in 0.5 M perchloric acid with a glass-Teflon homogenizer and periodically vortexed on ice and then centrifuged at 5,000 g for 5 min. Homogenates were neutralized with 2 M KHCO₃, and the supernatant was taken for glucose measurement using the Glucose (HK) Assay Kit according to 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 bichinonic acid protein assay. Glucose was assayed at identical time points as LDH.

Tdt-mediated dUTP nick-end labeling assay. Tissues from the in vivo ischemia-reperfusion model were collected ~2 h postinjection and fixed in 4% paraformaldehyde-0.01% glutaraldehyde in phosphate buffer for 30 min. Samples were cryoprotected in sucrose and then sectioned (14 µm thick) in the vertical plane and collected on precleared Superfrost Plus slides (LabServ).

Cell death was determined using the Tdt-mediated dUTP nick-end labeling (TUNEL) assay via the In Situ Cell Death Detection Kit (Roche Applied Science). Tissue sections were washed with PBS and then permeabilized with 0.1% Triton X-100-0.1% trisodium citrate in 0.1 M PBS for 5 min on ice. Sections were incubated with the TUNEL reaction mixture (1:17 enzyme-to-nucleotide ratio) for 30 min 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 Hardmounting medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

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

Immunohistochemistry. Retinal sections were blocked with 6% goat serum, 1% bovine serum albumin, and 0.1% (vol/vol) Triton X-100 for 1 h at room temperature and then incubated overnight with primary antibodies: anti-goat Brn-3A (catalog no. SC-31984, Santa Cruz Biotechnology; 1:200 dilution), anti-mouse glial fibrillary acidic protein (GFAP; catalog no. G3893, Sigma Aldrich; 1:1,000 dilution), anti-rabbit glutamate (catalog no. E100, Signature Immunologics; 1:500 dilution), anti-rabbit glutamine synthetase (GS; catalog no. MAB302, Millipore; 1:1,000 dilution). Primary antibodies were detected with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Mulgrave, VIC, Australia; 1:500 dilution). Sections were counterstained with DAPI and visualized on a confocal microscope as described for TUNEL labeling. Staining for each marker was performed in a single experimental run, and all images were obtained under identical brightness and contrast settings to enable intersample comparisons. Representative images in Fig. 5 were taken for brightness and contrast in Photoshop (version 8, Adobe, Mountain View, CA).

Image analysis. GFAP immunoreactivity was quantified by thresholding labeled images to remove background fluorescence (all pixels were valued between 0 and 255, and those valued at <20 were removed) and converting to binary images using ImageJ (National Institutes of Health). The nerve fiber layer (NFL) /ganglion cell layer (GCL) and inner plexiform layer (IPL) were delineated on the basis of DAPI labeling using the polygon tool in ImageJ, and GFAP is expressed as percentage of GFAP-positive pixels within the total area of the specific retinal layer using the measure tool in ImageJ.

Glutamate-labeled images were converted to grayscale and the total number of GS-positive pixels across the total retinal area was quantified. The GS pixel value was quantified within the NFL/GCL using the measure tool in ImageJ.

Glutamate immunoreactivity was quantified as described in Fig. 1. Retinal samples were colabeled with DAPI, glutamate, and GS and imaged on a confocal microscope as a 0.9-µm stack. The final image for quantification was composed of the pixels of maximum value among the five images (Fig. 1, step 1). The image was separated into individual channels (Fig. 1, step 2), and the GS images were thresholded and converted to binary images (Fig. 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 subtraction of the binary GS images from glutamate images using the Image calculator tool in ImageJ so that only glutamate immunoreactivity colocalized within GS labeling was visible (Fig. 1, step 4). The reverse subtraction was performed to give glutamate immunoreactivity in non-Müller cell space (i.e., neurons and other glia). Glutamate immunoreactivity of all cells in the sample was based on the unmodified glutamate image. Glutamate images were segmented into IPL and GCL/NFL on the basis of DAPI-labeled images. Glutamate within a region of interest was measured as mean pixel value (range 0–255) per square micrometer of area analyzed using the measure tool in ImageJ. For each retinal area, five retinae were assessed, and a total area of ≥50,000 µm² was assessed per retina. Immunoreactivity was then normalized to the glutamate content of the control eye treated with saline. Glutamine immunoreactiv-
Fig. 1. Image processing for glutamate immunoreactivity measurements. Retinal samples labeled with 4',6-diamidino-2-phenylindole (DAPI), glutamate, and glutamine synthetase (GS) were imaged (step 1) and then separated into individual red, green, and blue channels (step 2). GS-labeled images were thresholded to remove background fluorescence, and the remaining GS-immunoreactive pixels were 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 (step 3) to give glutamate immunoreactivity within GS pixels for glutamate content of Müller cells (step 4). Images generated from reverse subtraction (i.e., glutamate not colocalized with GS) were used to quantify glutamate content of non-Müller cells (step 4, left image). Images in step 4 were separated into the inner plexiform layer (IPL) and the ganglion cell layer/nerve fiber layer (GCL/NFL) using the DAPI image from step 3 before quantification. Scale bar = 20 μm.
ity was determined in an identical manner. For glutamate-to-glu-
tamine ratios, the pixel values were normalized to total GS pixels in
each sample, and the mean of each treatment group was compared for
the final ratio. All images used for comparative analysis were stained,
imagined, and quantified under identical conditions in a single exper-
imental run so that the net pixel value across samples could be
estimated.

Electroretinograms. Electroretinograms (ERGs) were used to de-
termine 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-wave and the b-wave, called oscillatory
potentials (30).

ERGs were conducted 1 h after injection of saline or vinpocetine (25
h postischemia) 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 was
placed in the tail (Grass Technologies, West Warwick, RI). Rats were
exposed to single 1-ms light flashes of increasing intensity (3.9 to 2.1
log cd·s⁻¹·m⁻²) from a Ganzfeld integrating sphere (Photometric Solu-
tions International, Huntingdale, VIC, Australia). Responses were re-
corded as the average of three flashes at each intensity at 4 kHz with
band-pass settings of −3 dB at 0.3–1,000 Hz (ML785, Powerlab/8SP,
AD Instruments; ×1,000 amplification).

ERG data were plotted and analyzed using the Michaelis-Menten
function of the amplitude-intensity relationship of the mixed cone-and
rod response (41). The amplitude of the a-wave was measured as the
minimum value of the ERG waveform trough, and the amplitude of the
b-wave was measured as the value from the 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- and b-wave peaks.

Metabolic inhibition assay. Pharmacological inhibition of retinal
metabolism was assayed based on the method described by Bui et al.
(15). Normal, dark-adapted, 6-wk-old Sprague-Dawley rats (n = 5)
were deeply anesthetized, and L-methionine sulfoximine (MSO;
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⁻¹·m⁻²) 40 min postinjection. At 90 min postinjection, 100 µM
vinpocetine was intravitreally injected into the left eye, and an equal
volume of saline was injected into the right eye. A second ERG
recording was obtained 70 min after vinpocetine treatment. ERG a-
and b-wave peaks were plotted relative to baseline values.

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
(control vs. treated) comparisons. Values are means ± SE. All data were
confirmed for normality using the Kolmogorov-Smirnov normality test
with Dallal-Wilkinson-Lillie for α = 0.05.

RESULTS

Metabolic effects of vinpocetine on subsequent 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). Normal LDH activity of the retina was

![Graphs showing the effect of vinpocetine on retinal lactate dehydrogenase (LDH) activity during normoxia, hypoxia, hypoglycaemia, and ischemia.](https://example.com/graph.png)
LDH release into the incubation medium (Fig. 2A). Comparatively, hypoxia, hypoglycemia, and ischemia (Fig. 2, B–D) significantly increased LDH activity in the retina (P < 0.05 for all insults, by Student’s t-test). These insults also significantly increased LDH release into the incubation medium (P < 0.001 for all insults, by Student’s t-test) compared with normoxia.

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

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 investigated these changes using an in vivo ischemia-reperfusion model to identify possible mechanisms for the actions of vinpocetine (14, 58). In the in vivo model, one eye was subjected to ischemia followed by 24 h reperfusion. Contralateral eyes were used as controls. After reperfusion, an intravitreal injection of saline or 100 μM vinpocetine was administered. Contralateral eyes received identical injection volumes.

LDH activity in contralateral control eyes was comparable to the ex vivo normoxia model (P = 0.5, by Student’s t-test; Fig. 3A). LDH activities were significantly higher in ischemia-reperfusion than control eyes (P < 0.05, by Student’s t-test). Vinpocetine had no effect on control eyes but significantly reduced LDH activity of eyes exposed to ischemia-reperfusion to normal levels (P < 0.001, by Student’s t-test; Fig. 3A).

We hypothesized that the effects of vinpocetine on retinal metabolism may involve glucose, as vinpocetine increases glucose levels in ischemic brain tissue (28, 55, 62). We found no difference in glucose concentration between saline-treated control eyes and saline-treated eyes exposed to ischemia-reperfusion (P = 0.2, by Student’s t-test; Fig. 3B). Vinpocetine significantly increased glucose concentration in control and ischemic eyes [P < 0.05 (control) and P < 0.01 (ischemia), by Student’s t-test]. Elevated glucose was also observed with vinpocetine treatment of the ex vivo normoxia (P < 0.01, by Student’s t-test; 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 GCL, suggesting loss of ganglion cells and/or displaced amacrine, glial, and endothelial cells (Fig. 3, C and D). In contrast to the ex vivo model, vinpocetine treatment significantly decreased cell death in the GCL 2 h after administration (P < 0.05, by Student’s t-test), possibly
due to differences in the experimental timelines of vinpocetine administration between the in vivo and ex vivo models. Few TUNEL-positive cells were present in the GCL of contralateral control eyes with and without vinpocetine treatment (data not shown).

Effect of vinpocetine on Müller cells. We suspected that 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 GFAP (12). GFAP was mostly restricted to the NFL and GCL of control eyes treated with saline or vinpocetine (Fig. 4, A–C). GFAP was significantly elevated in eyes exposed to ischemia-reperfusion, with immunoreactivity extending from the NFL to the IPL (R < 0.001, by Student’s t-test). Vinpocetine reduced GFAP immunoreactivity in eyes exposed to ischemia-reperfusion, but these levels were still significantly greater than in control eyes (R < 0.001 (GCL) and R < 0.05 (IPL), by Student’s t-test). Müller cells were also assessed for the established Müller cell marker GS. In all treatments, the total number of GS pixels remained constant, suggesting that 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 that the level of enzyme was reduced (R < 0.05, by Student’s t-test; Fig. 4D). Vinpocetine had no effect on GS pixel value in control eyes or eyes exposed to ischemia-reperfusion (R = 1.0 (control) and R = 0.9 (ischemia), by Student’s t-test).

We further investigated the effects of vinpocetine on Müller cells by assessing changes to a key biochemical pathway of the cell: conversion of glutamate to glutamine (11). This pathway is closely linked to energy metabolism (reviewed in Ref. 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. Semiquantitative analysis of glutamate immunoreactivity (Figs. 5 and 6) showed a significant increase in glutamate in the IPL following ischemia-reperfusion (R < 0.05, by Student’s t-test; Fig. 6A). Vinpocetine reduced the glutamate level of the IPL to that of the control eye (R = 0.6, by Student’s t-test). 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 within Müller cells, we segmented the retinal space into Müller and non-Müller cells on the basis of positive and negative GS immunoreactivity (Fig. 1). Glutamate immunoreactivity was significantly elevated in the Müller cell processes in the IPL (R < 0.01, by Student’s t-test; Fig. 6C) and slightly elevated in the end feet (R = 0.1; Fig. 4).
following ischemia-reperfusion. Vinpocetine reduced glutamate immunoreactivity of ischemic Müller cell processes in the IPL to levels comparable to control eyes ($P = 0.6$ for processes, by Student’s $t$-test).

Glutamate metabolism was not altered in non-Müller cells of the GCL/NFL in ischemic eyes ($P = 0.7$, by Student’s $t$-test) or with vinpocetine treatment ($P = 0.7$, by Student’s $t$-test; Fig. 6E). However, visual inspection of immunolabeled 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 colocalizing glutamate immunoreactivity with the ganglion cell marker Brn-3A (Fig. 6F). Ischemia-reperfusion significantly increased glutamate content of ganglion cells ($P < 0.001$, by Student’s $t$-test), 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 end feet ($P < 0.01$, by Student’s $t$-test; Fig. 6G). Vinpocetine altered glutamine immunoreactivity in control and ischemic eyes, indicating that the drug affects multiple amino acids within the Müller cell (Fig. 6H). Comparison of the glutamate-to-glutamine ratio within Müller cell end feet 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).

**Effect of vinpocetine on retinal function during ischemia.** ERGs were performed in the in vivo ischemia-reperfusion-exposed 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. 7, A, C, and 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. 7, B–D). Vinpocetine treatment significantly improved the b-wave (inner retina) response of ischemic eyes to levels comparable to control eyes ($P < 0.001$, by 2-way ANOVA). Vinpocetine, however, did not change the a-wave (photoreceptor) response, as this remained significantly lower in ischemic than control eyes. The oscillatory potentials, which indicate amacrine cell function, were not altered under any treatments (Fig. 7E).

**Metabolic effects of vinpocetine occur in other models of metabolic insult.** Previous studies indicate multiple actions of vinpocetine, including regulation of cation entry through ionotropic glutamate receptors, during ischemia (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 MSO, which inhibits GS and impairs glutamate-to-glutamine conversion, resulting in a reduction of the b-wave amplitude over a period of 90 min (7, 15, 51). Impaired Müller cell metabolism was evident as a decrease in b-wave amplitude on ERG but no change in a-wave response, as this remained significantly lower in ischemic than control eyes. The oscillatory potentials, which indicate amacrine cell function, were not altered under any treatments (Fig. 7E).

![Fig. 6. Quantification of relative glutamate and glutamine immunoreactivity following vinpocetine treatment. A and B: glutamate content was determined in the IPL and GCL/NFL. C and D: glutamate immunoreactivity colocalized with GS was used to determine glutamate content of Müller cell (MC) inner retinal processes in the IPL and Müller cell end feet in the GCL/NFL. E: glutamate pixels not colocalized with GS were used to determine glutamate content in non-Müller cells of the GCL/NFL, which include ganglion cells, displaced amacrine cells, astrocytes, and microglia. F: glutamate content (GC) of ganglion cells alone determined by colocalization of glutamate with Brn-3A. G and H: glutamine immunoreactivity colocalized with GS was used to determine glutamate content of Müller cell end feet and compare ratio of mean glutamate to glutamine levels from D and G. Immunoreactivity is expressed relative to the control retina treated with saline in each condition. Dotted line indicates a relative value of 1 (baseline value) for comparison between data columns. All samples were stained and imaged under identical conditions in a single experimental run so that relative glutamate immunoreactivity could be determined between samples. Values are means ± SE. Significantly different from control saline: $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ (by Student’s $t$-test).](http://ajpcell.physiology.org/doi/10.1152/ajpcell.00291.2014)
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 LDH activity of ischemic retinae to normal levels and increased glucose availability. Vinpocetine’s actions on glucose also likely explains its ability to modify glutamate homeostasis in ischemic Müller cells. Other actions of vinpocetine postischemia 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 that the drug may be effective in treatment of ischemia in the retina and other parts of the central nervous system.

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 that 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 lactate-pyruvate reaction 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 the idea that vinpocetine has a more permanent effect on glucose metabolism (29, 61).

Vinpocetine modulates retinal metabolism following ischemia. 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 that it is an indicator of retinal stress (1, 2, 8, 68, 69, 73, 75). Vinpocetine reduced LDH activity in the ischemic retina, although it also increased glucose levels. Vinpocetine acts similarly in the brain, reducing LDH activity in damaged brain tissue (21), despite improvement of oxygen and glucose levels (28, 55, 62). This suggests that vinpocetine may lower metabolic demand in ischemia via mechanisms other than modulation of glucose metabolism. Vinpocetine may increase the availability of other metabolites, as its actions in the MSO-inhibited retina were 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 that the drug’s metabolic actions are primary.

**Vinpocetine modifies Müller cell function following ischemia.** 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 that vinpocetine slightly lowered GFAP immunoreactivity in ischemia-reperfusion, suggesting that 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-to-glutamine ratios were elevated following ischemia-reperfusion. Vinpocetine lowered Müller cell glutamate-to-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 that the drug may not regulate amino acid homeostasis by direct action 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 that internal stores of glucose could reduce glutamate accumulation in postmortem ischemic Müller cells.

**Vinpocetine improves retinal function postischemia.** Our ERG studies showed, for the first time, that vinpocetine improves retinal function following ischemia-reperfusion. 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 showed 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 in the middle layer of the 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 (PDE) 1 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 explain recovery of the b-wave by vinpocetine 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 subsequent treatment with PDE5.
Vinpocetine’s effect on the a-wave is also likely to be transient, as no anatomic damage, such as photoreceptor death, was observed. As ERGs represent a more sensitive technique for detection of ischemia damage than does morphological examination, future studies with longer observational time frames may be needed to quantify the full extent of vinpocetine’s actions on retinal metabolism during ischemia.

**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 postischemia, likely through its actions on retinal glutamate receptor activation. These results indicate that vinpocetine has potential functional and neuroprotective applications in the treatment of ischemia in the retina and possibly other central nervous system tissues.

**ACKNOWLEDGMENTS**

We thank Sarah Ready, Christopher Edwards, Neda Nikanjam, Nawras Nabhani, and Yea-Seul Shin for conducting the preliminary LDH experiments and Stephen Mulcock, Jennifer Du, Lucia Lin, and Yang Wang for conducting the preliminary ERGs.

**GRANTS**

This work was supported in part by Auckland Medical Research Foundation Grant 1109007, New Zealand Optometric Vision Research Foundation Grant 3620315, National Health and Medical Research Council of Australia Grant 1099342, and University of New South Wales Grant PS35430.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

L.N.-S., B.J.O., M.T., C.X.G., M.K., and M.L.A. performed the experiments; L.N.-S., B.J.O., M.T., C.X.G., M.K., and M.L.A. analyzed the data; L.N.-S., B.J.O., M.T., C.X.G., M.K., and M.L.A. interpreted the results of the experiments; L.N.-S. and M.L.A. prepared the figures; L.N.-S. and M.L.A. drafted the manuscript; L.N.-S., M.K., and M.L.A. approved the final version of the manuscript; B.J.O. and M.L.A. developed the concept and designed the research.

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