INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

**Visual System Pathology Caused by Chronic Cerebral Hypoperfusion:
Loss of Pupillary Reflex, Retinal and Optic Nerve Degeneration,
and the Role of Light Toxicity**

by

William Dale Stevens

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

**Master of Science
(Specialization in Neuroscience)**

**Department of Psychology
and
the Carleton Institute of Neuroscience**

**Carleton University
Ottawa, Ontario
September, 2000
© 2000 William Dale Stevens**

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file *Votre référence*

Our file *Notre référence*

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-57781-3

Canada

The undersigned recommend to the
Faculty of Graduate Studies and Research
Acceptance of the thesis:

"Visual System Pathology caused by Chronic Cerebral Hypoperfusion : Loss of Pupillary
Reflex, Retinal and Optic Nerve Degeneration and the Role of Light Toxicity"

submitted by

William Dale Stevens

in Partial fulfillment of the requirements for
the degree of Master of Science

Chair

Thesis Supervisor

Carleton University
September, 2000

ABSTRACT

Sprague-Dawley rats underwent permanent bilateral ligation of the common carotid arteries (2VO) (n=63) or sham surgery (n=20). Half of the rats were post-surgically housed in constant darkness, the other half in a standard 12-hour light/dark environment. Rats were sacrificed at 3, 15, and 90 days post-surgery. 2VO resulted in loss of the pupillary reflex to light in approximately 58% of rats within 7 days post-2VO. Gallyas silver staining revealed ongoing fibre degeneration of the optic nerve (ON) of eyes that lost the reflex (2VO-noflex) at 3, 15, and 90 days post-2VO. Stereology based counting techniques indicated a reduction of cells of the retinal ganglion cell layer in 2VO-noflex eyes by 15, and 90 days post-2VO relative to eyes that retained the reflex (2VO-flex and sham). By 90 days, the 2VO-noflex eyes showed an absence of Thy-1 immunoreactivity within the retina, and thinning of the inner and outer plexiform layers. Severe loss of photoreceptors in 2VO-noflex eyes occurred in the light condition only. It was concluded that 2VO likely resulted in ischemic damage to the ON, causing loss of pupillary reflex and death of retinal ganglion cells in a subset of rats (~58%). Subsequent light toxicity resulted in death of the photoreceptors in these eyes.

ACKNOWLEDGEMENTS

My first thanks go to my Mom and Dad. Thank you for having always supported me in everything I have done, in every way I've needed. You have given me the opportunity to follow my dreams, and the means to strive for the limits of my potential. Thank you for always being there for me.

I would also like to extend my warmest gratitude to the amazing team of people I have been fortunate enough to call my labmates for the last four years. Thanks Maxine for your friendship. Thanks Nicole, for teaching me what you could, for laughing with me, and for being such a bold gambler. And immeasurable thanks to you Teresa, for teaching me just about everything I needed to know to get here, and for all of your assistance with this project. I never could have done this without you.

Most of all, I would like to express my deepest respect, gratitude, and admiration for my advisor and mentor, Dr. Bruce Pappas. Thank you for your patience, your generosity, and your confidence in me. The last four years working under you have afforded me the opportunity to explore my ambitions, and gain the experience I needed to take the next step in my life. I move forward with the greatest of optimism and anticipation, but I will always look back with the fondest of memories. I hope one day to be as accomplished a man as you. Thanks Bruce.

TABLE OF CONTENTS

Title Page	i
Acceptance Sheet	ii
Abstract	iii
Acknowledgements	iv
Table of Contents	v
List of Tables	viii
List of Figures	ix
List of Appendices	xi
List of Abbreviations	xii
Introduction	1
Cerebral Blood Flow and 2VO	2
Retinal Blood Flow and 2VO	3
Behavioural Effects of 2VO	6
2VO and Vision	8
2VO and Retinal Pathology	12
Pupillary Reflex	18
Clinical Relevance of the 2VO Model	22
Summary and Rationale	25

Methods	28
Animals	28
Pupillary Reflex	28
Groups	29
Surgery	29
Tissue Preparation	31
Stereology	32
Thy-1	33
Retinal Sublayers	35
Optic Nerve	35
 Results	 37
Surgery and Pupillary Reflex	37
Retinal Ganglion Cell Layer	41
Stereology	41
Thy-1	45
Retinal Sublayer Pathology	52
Outer Nuclear Layer	52
Outer Plexiform Layer	59
Inner Nuclear Layer	59
Inner Plexiform Layer	59
Optic Nerve Pathology.....	70

Gallyas Silver Stain	70
GFAP Immunoreactivity	70
Discussion	82
Pupillary Reflex	82
RGCL Pathology	84
Retinal Sublayer Pathology	86
Outer Nuclear Layer	87
Outer Plexiform Layer	87
Inner Nuclear Layer	88
Inner Plexiform Layer	89
Optic Nerve Pathology	89
Gallyas Silver	89
GFAP Immunoreactivity	90
Suggestions for Future Research	91
Clinical Relevance	94
Conclusions	96
References	98
Appendix A	106
Appendix B	115

LIST OF TABLES

Table	Description	Page
1	Number of Animals per Group	30
2	Number of Animals per Reflex Group	38
3	Number of Eyes per Group Used for Histological Analysis	42

LIST OF FIGURES

Figure	Description	Page
1	A: Sagittal representation of the arteries originating at the aorta which service the eyes and brain	4
	B: Bottom projection of the arteries servicing the eyes and brain	4
2	Representation of the cells and layers of the retina	13
3	Detailed circuit diagram of the pupillary reflex pathway	19
4	Number of 2VO animals with no loss, unilateral loss, or bilateral loss of pupillary reflex	39
5	Digital images of sections of retina stained with hematoxylin	43
6	Stereological population estimates of neurons in the RGCL, all subgroups	46
7	Stereological population estimates of neurons in the RGCL, group×day	48
8	Digital images of sections immunohistochemically stained for Thy-1	50

9	Percent area of retina showing Thy-1 immunoreactivity, all subgroups	53
10	Percent area of retina showing Thy-1 immunoreactivity, group×day	55
11	Thickness of the outer nuclear layer	57
12	Thickness of the outer plexiform layer, all subgroups	60
13	Thickness of the outer plexiform layer, group×day	62
14	Thickness of the inner nuclear layer	64
15	Thickness of the inner plexiform layer, all subgroups	66
16	Thickness of the inner plexiform layer, group×day	68
17	Digital images of optic nerve cross sections stained with Gallyas silver and for GFAP immunoreactivity	71
18	Percent area of optic nerve stained with Gallyas silver, all subgroups	73
19	Percent area of optic nerve stained with Gallyas silver, group×day	75
20	Percent area of optic nerve showing GFAP immunoreactivity, all subgroups	78
21	Percent area of optic nerve showing GFAP immunoreactivity, group×day.....	80

LIST OF APPENDICES

Appendix	Description	Page
A.1	ANOVA Summary Table for Post-Surgical Reflex Condition	106
A.2	ANOVA Summary Table for Neuron Population Estimates of the RGCL.....	107
A.3	ANOVA Summary Table for Thy-1 IR in the RGCL	108
A.4	ANOVA Summary Table for Outer Nuclear Layer Thickness	109
A.5	ANOVA Summary Table for Outer Plexiform Layer Thickness	110
A.6	ANOVA Summary Table for Inner Nuclear Layer Thickness	111
A.7	ANOVA Summary Table for Inner Plexiform Layer Thickness	112
A.8	ANOVA Summary Table for Gallyas Silver Staining in the Optic Nerve	113
A.9	ANOVA Summary Table for GFAP IR in the Optic Nerve	114
B	Summary Table for Scores on the Dependent Measures for Subgroups of Sham Eyes Which Made Up the CON Group.....	115

LIST OF ABBREVIATIONS

2VO	two vessel occlusion
AD	Alzheimer's disease
AF	amaurosis fugax
ang	angular artery
ANOVA	analysis of variance
BICL	bilateral internal carotid artery ligation
CBF	cerebral blood flow
cctd	common carotid artery
CE	coefficient of error
CNS	central nervous system
CON	control
DAB	3,3'-diaminobenzidine
ectd	external carotid artery
E-W	Edinger-Westphal nucleus
eoph	external ophthalmic artery
ERG	electroretinogram
faci	facial artery
flex	pupillary reflex intact
GFAP	glial fibrillary acidic protein
ictd	internal carotid artery
i.m.	intramuscular

IOP	intraocular pressure
INL	inner nuclear layer
i.p.	intraperitoneal
IPL	inner plexiform layer
ioph	internal ophthalmic artery
IR	immunoreactivity
MRI	magnetic resonance imaging
MWM	Morris water maze
NFL	nerve fibre layer
noflex	no pupillary reflex
ONL	outer nuclear layer
OPL	outer plexiform layer
OPN	olivary pretectal nucleus
PBS	phosphate buffered saline
PRL	photoreceptor layer
ptgpal	pterygopalatine artery
RAM	radial arm maze
RBF	retinal blood flow
RGC	retinal ganglion cell
RGCL	retinal ganglion cell layer
vert	vertebral artery
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

INTRODUCTION

Various methods of experimentally induced ischemia in rats have been used to model human cerebrovascular disorders. Induction of ischemia is usually achieved by transient occlusion of one or more of the ascending arteries servicing the brain. Acute ischemia models of this type are useful for exploring pathology associated with myocardial infarction and stroke in humans. Less common are experimental models which explore the effects of chronic reduction of cerebral blood flow (CBF), despite the parallels to cerebrovascular insufficiency associated with carotid artery disease, multi-infarct dementia, as well as age-related and Alzheimer-type dementia. A commonly used experimental technique for this purpose is two vessel occlusion (2VO) which involves permanent bilateral ligation of the common carotid arteries in rats.

The behavioural correlates and cerebral neuropathological effects associated with 2VO in rats have been extensively explored. However, the related effects of retinal ischemia and the potential for visual system pathology in the 2VO model have rarely been considered. Because retinal blood flow (RBF) originates almost exclusively from the common carotid arteries (Scremin, 1995), it is essential to consider the effects of ligation of these arteries on the visual system in rats. Indeed, evidence in the literature suggests that the visual system is compromised by 2VO (Ohta et al., 1997; Tanaka et al., 1996; Yamamuro et al., 1996). Recent research in this laboratory has revealed that 2VO can cause permanent loss of the pupillary reflex, retinal degeneration, and visual impairment in Sprague-Dawley rats (Davidson et al., 2000). The purpose of this study is to investigate the temporal emergence of retinal degeneration and pupillary dysfunction in 2VO rats, as

well as the possible contribution of light toxicity to these deficits.

Cerebral Blood Flow and 2VO

The common carotid arteries, along with the vertebral arteries, make up the four major ascending arteries which supply blood to the brain. Ligation of the common carotid arteries results in reduced CBF which tends to abate over the weeks thereafter.

By measuring the difference between arterial and venous blood concentrations of CO₂ and O₂, it was estimated that the overall reduction of CBF shortly after 2VO was approximately 50% (Eklöf and Siesjö, 1972). This 50% reduction of CBF was confirmed by the same researchers using the fully quantitative [¹⁴C]-iodoantipyrine autoradiographic technique (¹⁴C) (Eklöf and Siesjö, 1973). Reductions of CBF to approximately 40% of control levels were detected specifically in the cortex and thalamus within 5 hours of 2VO using the hydrogen clearance method by Fujishima et al. (1981). Using the ¹⁴C method, local CBF levels were quantified in 24 anatomically discrete regions in unanesthetized rats at 2.5 hours and 1 week after 2VO (Tsuchiya, et al., 1992). Local CBF reductions noted at 2.5 hours post-occlusion were: 40% in the caudate nucleus, 25-39% in cortical regions, 52% in the hippocampus, 39% in the lateral geniculate body, and 45-85% in the thalamic regions, medial geniculate body, hypothalamus, amygdala, septal nucleus, nucleus accumbens, globus pallidus, substantia nigra, superior colliculus, corpus callosum and internal capsule. Although measurements at 1 week post-2VO indicated that CBF was still significantly reduced, there was an amelioration of this in almost all brain regions. Other studies have shown similar results. CBF is generally reduced by approximately 50%

shortly after 2VO, and slowly increases to approximately 70% of normal control levels by 7 days following 2VO, where it remains for at least several months (Ohta, et al., 1997; Tanaka et al., 1996).

The progressive reperfusion that is consistently shown to follow 2VO may be facilitated by the formation of collateral arteries shunting the occlusion. Oldendorf (1989) found changes in the posterior circle of Willis and the basilar and intracranial vertebral arteries following 2VO. Progressive alterations of vascular morphology resulted in basilar and vertebral artery tortuosity, enlargement, and duplication of the vertebro-basilar junction. Some of these changes regressed towards normal morphology by 15 weeks post-ligation, accompanying the appearance of multiple collateral vessels within the soft tissues of the neck, shunting the ligation.

Retinal Blood Flow and 2VO

The common carotid artery is essential in supplying blood to the eye and its components in the rat (for review see Scremin, 1995; Greene, 1935). Arterial supply begins at the common carotid artery, and ends at a converging multi-arterial network at the back of the eye (Figure 1). The common carotid artery splits, giving rise to the external and internal carotid arteries in this order. The pterygopalatine artery originates from the internal carotid artery and diverges towards the eye, ultimately supplying it with the majority of its blood supply through subsequent arteries.

Blood from the pterygopalatine artery reaches the eye via the external ophthalmic artery. The internal ophthalmic and trigeminal arteries stem from the internal carotid as

Figure 1A: Sagittal representation of the arteries servicing the eyes and brain originating at the aorta. ★ represents approximate point of carotid ligation. Figure adapted from Scremin (1995).

Figure 1B: Bottom projection of the arteries servicing the eyes and brain. Figure adapted from Scremin (1995).

Artery names in **bold** are mentioned in the text. Abbreviations: acer, anterior cerebral artery; afor, alar foramen; **ang, angular artery**; azac, azygos anterior cerebral artery; bas, basilar artery; bcph, brachiocephalic trunk; **cctd, common carotid artery**; dpal, descending palatine artery; dsp, dorsal spinal artery; **ectd, external carotid artery**; **eoph, external ophthalmic artery**; **faci, facial artery**; **ictd, internal carotid artery**; **ioph, internal ophthalmic artery**; iorb, infraorbital artery; ling, lingual artery; mcer, middle cerebral artery; olfa, olfactory artery; pcer, posterior cerebral artery; pcom, posterior communicating artery; ptg, pterygoid artery; **ptgpal, pterygopalatine artery**; sbcl, subclavian artery; scba, superior cerebellar artery; vbr, vibrissal arteries; **vert, vertebral artery**; vsp, ventral spinal artery.

well, at a position close to the back of the eye. These three arteries (the external ophthalmic, internal ophthalmic, and trigeminal) converge at the back of the eye. The eye also receives blood via the angular artery, which is supplied by the external carotid artery via the facial artery. The majority of RBF, transported by the retinal artery (nasal and temporal branches), arrives via the external ophthalmic artery (Scremin, 1995). Because RBF normally originates from the common carotid artery, retinal ischemia would seem an obvious result of 2VO. It has been noted however that RBF may increase over time following 2VO, supplied from the vertebral arteries via the circle of Willis (Block, et al., 1992).

It is important to note that the retina is particularly vulnerable to ischemia due to the structure of the retinal capillaries. As compared to surrounding structures such as the choroid and ciliary processes, retinal capillaries are much smaller, with a calibre of 5-7 μ m, and are sparsely distributed leaving large vessel-free spaces across the retina (Funk, 1997). This capillary structure results in a relatively low total blood volume, although the blood flow velocity is high. Whereas surrounding structures are supplied with an excess of nutrients via blood flow, the retina is not, as indicated by a large arteriovenous pO₂ difference (38%) (Funk, 1997). Limited blood supply due to sparse capillary distribution, while optimizing visual function, renders the retina intolerant of hypoperfusion.

Behavioural Effects of 2VO

Chronic ischemia in the rat, as in 2VO, results in performance deficits on spatial memory tasks such as the Morris water maze and various configurations of radial arm

mazes. These tasks have traditionally been used to assess cognitive function such as memory and learning ability (task acquisition). Performance deficits on these tasks have been attributed to cognitive deficits, presumably associated with cerebral pathology. It has rarely been noted that all of these tasks are visually-guided tasks which may be dependant to some degree on visual function. The potential role of visual dysfunction in maze performance following 2VO may confound inferences as to the effects of chronic ischemia on learning and memory.

The radial arm maze (RAM) (Olton and Papas, 1979) has been used in various ways to assess task acquisition and memory in 2VO rats. Rats subjected to 2VO show a consistent behavioural profile which reflects performance deficits as compared to control animals. First, maze-naïve rats tested after 2VO tend to show higher error rates and an inability to acquire the task as compared to control animals (Ni et al., 1995a; Ni et al., 1994). Second, rats that are pre-trained on a RAM task prior to 2VO tend to display deficits which are not apparent immediately following 2VO, but which emerge and increase over time (Pappas et al., 1996; Ni et al., 1994).

The Morris water maze (MWM) is also commonly used to assess spatial memory and learning. The task often involves repeated escape trials where rats must learn and recall the location of a submerged platform at a constant position within a pool of water. Increased platform location latencies are presumed to reflect a cognitive deficit (Morris et al., 1982). A subsequent probe trial is often administered wherein the rat is placed in a pool containing no platform, then proportion of path length or time spent in the quadrant of the previous platform location is calculated. Significantly increased time or path length

spent in this quadrant reflects a spatial bias, the absence of which is presumed to reflect a reference memory deficit (Morris et al., 1982). 2VO rats are consistently impaired on the MWM, showing increased platform location latencies and reduced spatial bias during probe trials (De Jong et al., 1999; Bennett et al., 1998; Ohta et al., 1997; Pappas et al., 1996). Further, exposing the rats to pre-surgical training does not appear to alter performance following 2VO (de la Torre et al., 1997).

While the patterns of behavioural deficits occurring on these mazes are relatively well established and consistent across studies, the cause of these deficits is less clear. Although these tasks have traditionally been used to assess cognitive performance, they all rely to some extent on visual ability. For example, it has been shown that deficits on the MWM correlated with the degree of photoreceptor degeneration within the retina of rats (O'Steen et al., 1995; Spencer et al., 1995). There is an emerging body of evidence which indicates that 2VO does result in visual dysfunction, and that this causes performance deficits on visually-guided behavioural tasks.

2VO and Vision

Several studies have reported behavioural deficits in 2VO rats which more directly indicate the presence of visual dysfunction. A brightness discrimination task which required rats to press a lever to receive a food reward in response to the presentation of a correct light stimulus was conducted by Yamamuro et al. (1996). In this experiment, control rats displayed an increasing rate of correct responses over time as they acquired the task. Conversely, 2VO rats were unable to acquire the task despite 30 days of post-

surgical training. The ratio of correct responses to total lever presses by the 2VO rats was not significantly above chance levels (~50%). Similar results were shown in another study where 2VO rats failed to acquire this task during 60 consecutive days of testing commencing 6 weeks after 2VO (Tanaka et al., 1996).

A recent study by Ohta et al. (1997) compared the behavioural effects of the 2VO model to the effects of a novel chronic ischemia model where the bilateral internal carotid arteries were permanently ligated (BICL). In the BICL procedure, each internal carotid artery was ligated at a point superior to the bifurcation of the pterygopalatine artery which is the primary source of blood flow to the ipsilateral eye. At 2 weeks and 3 months post-surgery, the 2VO rats were impaired on the MWM, while the BICL rats were not. 2VO rats showed significantly increased platform location latency and reduced spatial bias during the probe trial as compared to both the BICL and control animals which did not differ. On an 8-arm RAM, both BICL and 2VO rats were impaired in that they committed more errors than control animals. However, the 2VO animals were more severely impaired, committing significantly more errors than the BICL animals.

There are at least two possible explanations for the difference in behavioural impairment between the two ischemic groups observed in the study by Ohta et al. (1997). First, quantitative differences in the reduction of CBF may have resulted in varying degrees of cerebral pathology and thus cognitive impairment. Quantification of regional CBF at 2, 10, and 90 days post-ligation using the coloured microsphere method indicated that the 2VO group suffered more severe reductions than the BICL, group which suffered reductions only in comparison to the control group (CBF levels were not significantly

different at 3 months post-ligation). A second explanation would be that the BICL procedure, by ligating above the bifurcation of the pterygopalatine artery, did not attenuate RBF as in the 2VO model where this artery is effectively ligated along with the common carotid artery. It is thus suggested that visual dysfunction may have been to some degree responsible for the resulting maze deficits, and that the absence of retinal ischemia after BICL may have spared visual function. This latter explanation is supported by the finding that 2VO animals, and not BICL animals, showed abnormal circadian rhythms of spontaneous motor activity. The 2VO rats displayed a circadian cycle which was not synchronized with the light/dark cycle suggesting that 2VO, but not BICL rats, were visually impaired.

Perhaps the most compelling evidence that 2VO can result in visual dysfunction has emerged from recent experiments in this laboratory (Davidson et al., 2000) which show for the first time that 2VO causes permanent loss of the pupillary reflex to light in some rats. Further, retinal neuropathology was present only in this sub-group of rats which lost the pupillary reflex (2VO-noflex) and was not apparent in 2VO animals which retained the reflex (2VO-flex). Finally, the 2VO-noflex rats, and not 2VO-flex rats, were impaired on visually-guided behavioural tasks.

It has been determined in our laboratory that approximately 2/3 of Sprague-Dawley rats (Charles River Laboratories, Montreal) subjected to 2VO show abnormalities of the pupillary reflex to light. The behavioural correlates of this pupillary dysfunction were investigated in two experiments (Davidson et al., 2000). 2VO rats in one experiment were pre-trained on a 10-arm RAM until they met a strict performance criterion prior to

surgery. Testing resumed 3-4 days later for 16 weeks, ceased for 7 weeks and then resumed for 1 week. The 2VO-noflex rats committed more working memory errors than the control rats, while the 2VO-flex rats did not differ from controls. Further, the 2VO-noflex rats committed more reference memory errors than both the control group and the 2VO-flex group which did not differ. The same rats were tested on a visually-cued version of the MWM, 9 trials per day for 10 days commencing at post-operative day 220. Rats were required to locate a submerged platform which was randomly positioned between trials, but always positioned immediately beneath one of two different large visual cues suspended above the water. The 2VO-noflex rats took significantly longer to find the submerged platform than both the 2VO-flex and control groups which did not differ. These data indicate that loss of pupillary reflex correlated with deficits on visually-guided mazes.

The second experiment in this study (Davidson et al., 2000) tested rats on a novel visual/tactile discrimination maze. The maze consisted of a 12-arm RAM. The central hub was enclosed by a 30 cm wall with remotely controlled, sliding guillotine-style doors allowing entry into each arm. Each arm could be cued as black or white. To provide the tactile cue, the floor of each arm was lined with an interchangeable strip of polyurethane matting, either rough or smooth surfaced. The rats received a food reward for entering arms which contained the combination of the correct visual and tactile cues. Six of the twelve arms contained the correct combination of visual and tactile cues and were baited with a food reward, three arms contained an incorrect visual cue (unbaited), and three arms contained an incorrect tactile cue (unbaited). The configuration of these arms was

changed between sessions. For each session, rats were allowed to enter baited arms only once, after which access was blocked by the sliding door. The unbaited arms, which contained one incorrect cue either visual or tactile, remained accessible throughout the entire session. In this task, rats could commit tactile errors by entering arms containing an incorrect tactile cue, or visual errors by entering arms containing incorrect visual cues. Rats were trained to a strict performance criterion prior to surgery, and were tested approximately every 2nd-3rd day until 62 days post-surgery. All rats displayed very few tactile errors and there were no differences in the number of tactile errors committed by the control, 2VO-flex, and 2VO-noflex groups. Conversely, the 2VO rats committed more visual errors than controls, with the 2VO-noflex group committing the most visual errors at all time points.

2VO and Retinal Pathology

A limited number of studies have investigated neuropathology of the retina associated with 2VO. These studies have identified an immediate abnormality of retinal transmission and later appearing progressive retinal degeneration. Recent research in this laboratory has revealed that severe retinal degeneration appears to correlate with loss of the pupillary reflex.

The mammalian retina is organized in well defined layers which form an approximate hollow hemisphere encompassing the back of the eye (for review see Kandel et al., 1995; Wässl and Boycott, 1991). Briefly, the retina consists of the following layers, starting at the back and working forward (from outer to inner layers) (Figure 2). The

Figure 2: Representation of the cells and layers of the retina. Abbreviations: PRL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer; NFL, nerve fibre layer.

photoreceptor layer is immediately at the back of the retina where signal transduction begins. In the rat retina, this layer consists of both rods and cones, although cones make up only about 0.85% of all photoreceptors (Sefton and Dreher, 1995). The outer nuclear layer (ONL) is a layer of densely packed cell bodies which are the photoreceptor perikaryon. The outer plexiform layer (OPL) is the site of synaptic connections between the photoreceptor, bipolar and horizontal cells. The inner nuclear layer (INL) is another layer of densely packed cell bodies of three types. The horizontal cell bodies are located on the outer margin of the INL, bipolar cell bodies are located throughout the INL, and amacrine cell bodies are located towards the inner margin of the INL. The inner plexiform layer (IPL) is the site of interconnections of the bipolar, amacrine, and ganglion cells. The ganglion cell layer (RGCL) consists of the retinal ganglion cells (RGCs) as well as a population of amacrine cells which are referred to as displaced amacrine cells (Wässl and Boycott, 1991). The innermost layer is the nerve fibre layer (NFL) consisting of the axons of the RGCs which form the optic nerve when they leave the orbit. Astrocytes are present in the NFL which, along with Müller cells, are the only glial cells in the retina. The Müller cell bodies are located amongst the bipolar cells in the inner nuclear layer, while their processes span the entire retina.

Generally, information flows vertically from the photoreceptors to bipolar cells and to RGCs. Information flows laterally within the OPL as mediated by horizontal cells, and within the IPL as mediated by the amacrine cells (Kandel et al., 1995). Glutamate is the primary neurotransmitter involved in signal transduction within the retina (for review see Pourcho, 1996), although it is probable that every type of neurotransmitter found in the

central nervous system (CNS) may be found within the retina as well, mostly occurring within the more than 30 identified different types of amacrine cells (Wässl and Boycott, 1991). Release of glutamate by the photoreceptors is maximal in darkness, and is decreased by exposure to light, as these cells are hyperpolarized in response to light stimulation.

Abnormalities of retinal signal transduction have been shown to occur immediately following 2VO. Recording of the photopic electroretinogram (ERG) in response to light stimulation with a strobe showed a decrease in the amplitude of the b-wave during 2VO (Block et al., 1992). The ERG b-wave is generated to a great extent by the Müller cells and suppression of the wave amplitude is considered to be a sensitive indicator of ischemia. Initiation of the b-wave is thought to be caused by depolarization of on-bipolar neurons in response to light (Block et al., 1992). Longer periods of 2VO resulted in increased suppression of the ERG b-wave. At 45 minutes post-ligation, the b-wave amplitude of 2VO rats was reduced to 46% as compared to the control rats, while after 7 days of 2VO, the b-wave was abolished (Barnett and Osborne, 1995). After 3 and 9 months of 2VO, both the b-wave and the a-wave, which reflects activity of the photoreceptors, were completely abolished, with the exception that only slight traces of these waves occurred infrequently at the 3 month time point (Osborne et al., 1999).

Indications of retinal neural damage are evident at early time points following 2VO. Expression of retinal glial fibrillary acidic protein (GFAP), which is associated with gliosis within the Müller cells, was increased by 356% following 7 days of 2VO (Barnett and Osborne, 1995). Others have found evidence of degeneration within the optic nerve

and optic tract following 2VO as well (Wakita et al., 1995). At 7, 14, and 30 days following 2VO, rarefaction of white matter and increased activation of microglia/macrophages occurred within the optic nerve and optic tract (Wakita et al., 1998). Another study noted pronounced shrinkage of the optic nerves of 2VO rats occurring either bilaterally or unilaterally (Ohta et al., 1997). This laboratory has found evidence that apoptotic cell death of the photoreceptors appears to be maximal at 2 weeks following 2VO, while RGCs appear to suffer apoptotic demise beginning after 10 weeks of 2VO (unpublished results).

A recent study by Osborne et al. (1999) tracked the effects of 2VO on the retina to considerably longer time points and found remarkably subtle retinal degeneration. After 3 months of 2VO, visual inspection of retina by light microscopy revealed a moderate thinning of the overall retinal thickness which tended to be a result of somewhat thinner outer retinal regions. Retinal GFAP immunoreactivity (IR) was increased while markers used for a number of different retinal cell types showed no changes. After nine months of 2VO, the overall retinal thickness was further reduced with marked reductions in the outer regions, as indicated by reduced Ret-P1 staining indicating a loss of photoreceptors. No other significant reductions of other retinal cell types were noted.

The most substantial retinal degeneration associated with 2VO was demonstrated by Davidson et al. (2000). Retinal and optic tract degeneration were investigated in rats that were sacrificed at 300 days post-surgery. Quantification of the area stained with the Gallyas silver technique (Gallyas et al., 1990) and for GFAP indicated increased fibre degeneration and reactive astrogliosis respectively in the optic tracts of 2VO-noflex rats

as compared to both 2VO-flex and control groups. Further, 2VO-noflex rats had reduced cell density in the RGCL, and reduced overall layer thickness of the OPL and ONL relative to the 2VO-flex and control groups which did not differ on any of these measures. Thus 2VO causes pupillary reflex dysfunction which is highly correlated with retinal degeneration in a subset of rats.

Pupillary Reflex

The pupillary light reflex involves constriction of the pupil, which acts to control the amount of light entering the eye and reaching the retina, effectively limiting the potential for light toxicity to the photoreceptors (for review see Chan et al., 1995; Kandel et al., 1995) (Figure 3). Signal transduction in this reflex pathway begins with photoreceptors responding to light, ultimately activating RGCs. The RGC axons project bilaterally to the right and left olivary pretectal nuclei (OPN) which are located at the junction of the midbrain and thalamus, rostral to the superior colliculi. Axons from the OPN project bilaterally to the preganglionic parasympathetic Edinger-Westphal nuclei (E-W). The E-W nucleus lies adjacent to the oculomotor (cranial nerve III) nucleus and it projects axons which innervate the ciliary ganglion. Postganglionic axons from the ciliary ganglion innervate the pupillary sphincter which is responsible for constriction of the pupil. The pupillary dilator muscle which is responsible for pupil dilation is innervated by sympathetic fibres from the superior cervical ganglion. During light stimulation, the parasympathetic action is initiated and the sympathetic dilator system is inhibited resulting in pupillary constriction. The reverse of these actions occurs during pupil dilation.

Figure 3: Detailed circuit diagram of the pupillary reflex pathway. Abbreviations: OPN, olivary pretectal nucleus; EW, Edinger-Westphal nucleus; CG, ciliary ganglion; vLGN, ventral lateral geniculate nucleus; VC, visual cortex. Figure adapted from Chan et al. (1995).

Exposure of an eye to light causes constriction in that eye (direct response) as well as constriction in the contralateral eye (consensual response). It should be noted that the description of the pupillary reflex pathway provided is a simplified model of the actual system. Other structures within the CNS including the posterior commissure, ventral lateral geniculate nucleus, and inputs from the cortex are involved in mediation of the pupillary reflex as well (Chan et al., 1995; Legg, 1975).

The pupillary reflex has an ipsilateral and a contralateral pathway, giving rise to the direct and consensual responses. In the rat, the contribution of these two pathways is asymmetric, and differences exist between albino and pigmented rats (Chan et al., 1995; Sefton and Dreher, 1995). Ipsilateral projection of the RGC axons to the OPN has been estimated to be approximately 3-6% and 1.5-3% of total projection in pigmented and albino rats respectively. Despite the small proportion of ipsilateral projection to the OPN relative to contralateral axonal projection, the relative functional contribution of this pathway is much higher (Chan et al., 1995). The proportion of function achieved by the ipsilateral pathway is 29% (71% contralateral). Signal transduction between the OPN and E-W nuclei is bilateral as well, with 48% ipsilateral and 52% contralateral. Whereas pigmented rats display an equal pupillary response and threshold (intensity of light required to initiate response) for the direct and consensual responses, albino rats show a reduced consensual response with a higher threshold as compared to the direct response. This has been attributed to a reduced ipsilateral pathway in albino rats. Finally, albino rats display more variability in the degree of pupil constriction in response to light both within an individual and between individuals than their pigmented counterparts (Chan et al.,

1995). It should be noted that the bilateral structure of the pupillary reflex, crossing at two points, facilitates survival of the reflex in many cases of unilateral or even bilateral pathology such as lesions.

Clinical Relevance of the 2VO Model

2VO models the effects of the chronic cerebral hypoperfusion associated with several human disorders, including Alzheimer's disease (AD) (Crawford, 1998; Kalaria, 1996; Panza et al., 1996; Pietrini et al., 1996; Benson et al., 1983). A study using positron emission tomography revealed that AD patients suffered from reductions in regional cerebral glucose metabolism due to reduced CBF (Blesa et al., 1996). It has also been shown using single photon emission computed tomography that reductions of CBF correlate with the rate of cognitive decline in AD patients (Thome et al., 1996). One theory of AD etiology suggests that changes occurring within brain capillaries such as basement membrane thickening and amyloid deposition may result in low grade ischemia which can initiate a cascade of neuropathological events which cause AD (de la Torre, 1999; Kalaria and Hedera, 1995; de la Torre and Mussivand, 1993). It has recently been shown that age-related ultrastructural aberrations of the capillaries within the CA1 region of the hippocampus (a highly affected area in AD) of rats were exacerbated by 2VO (De Jong et al., 1999).

Several other correlates of AD are modelled in the 2VO rat as well. Abnormalities in acetylcholine levels (Kondo et al., 1996; Yamamuro et al., 1996; Ni et al., 1995b), increased GFAP IR, and loss of neurons primarily in the hippocampus (de la Torre et al.,

1992) are all factors which occur in both AD and 2VO. Stereology based counts of hippocampal CA1 neurons, a population of cells targeted by AD pathology, were recently conducted in this laboratory demonstrating a reduction of approximately 10% in 2VO rats relative to controls (unpublished results). Neuronal cell death associated with AD may be to some extent apoptotic (Su et al., 1994) as is the case in the ischemic rat brain (Bennett et al., 1998; Chen et al., 1997; Islam et al., 1995). As well, it was recently demonstrated that the 2VO model reproduces features of β -amyloid biogenesis characteristic of sporadic Alzheimer's disease (Bennett et al., 2000).

The visual system is targeted by the pathological progression of AD as well. Examination of the retinas of AD patients revealed degeneration primarily within the RGCL (Blanks et al., 1989). Although neurofibrillary tangles and neuritic plaques associated with AD were not present in the retina, vacuolated cytoplasm, shrunken somas, and cell loss or nuclear pyknosis were all detected within the RGCL. Within the central retina, a 25% decrease in the total number of neurons in the RGCL was noted (Blanks et al., 1996a). AD was also associated with an increase in GFAP IR, more extensive labelling of astrocytes in the RGCL, and increased labelling of Müller cell end-feet (Blanks et al., 1996b).

AD patients also show an exaggerated pupillary reaction to the administration of the cholinergic antagonist tropicamide. This has been attributed to selective targeting of the E-W nucleus by AD early in the course of the disease (Scinto et al., 1999). It was found that neurofibrillary tangles and neuritic plaques were particularly abundant in the E-W nucleus, while the adjacent somatic portion of the oculomotor complex was virtually

spared of pathology.

A number of other diseases involve visual pathology associated with retinovascular insufficiency such as HIV (Iragui et al., 1996; Yung et al., 1996), and diabetes (Bazan et al., 1997). The 2VO model is perhaps most relevant as a model for carotid artery disease in humans. In carotid artery disease, CBF is reduced by stenosis of the common or internal carotid artery as a result of atherosclerotic plaques occurring either unilaterally or bilaterally (Dugan and Green, 1991). Cognitive deficits have been associated with carotid artery disease as indicated by neuropsychological tests on parameters such as mental speed, learning, visuospatial abilities, verbal processing, and deductive reasoning (Taghavi and Hamer, 1995; Benke et al., 1991). Some studies have argued that reversal of cerebral ischemia by carotid endarterectomy can improve neuropsychological parameters (Kelly et al., 1980; Haynes et al., 1976), while others have shown only temporary benefits (Bennion et al., 1985) or no improvement at all (Casey et al., 1989; Parker et al., 1986).

Carotid artery disease often results in a number of disorders of the visual system. Amaurosis fugax (AF), probably the most common ocular symptom of carotid artery disease, is a temporary blindness resulting from arterial emboli or transient flow diminution (Ruben et al., 1990; Fisher, 1989). AF, or transient monocular blindness, following exposure to bright light may result from the inability of reduced RBF to meet increased metabolic demands of the retinal response to light (Dugan and Green, 1991). Progression of retinal pathology may involve venous stasis retinopathy and ocular ischemic syndrome which are associated with microaneurysms, neovascular glaucoma, and permanent blindness. It is significant to note that human carotid artery disease is associated with

pupillary reflex dysfunction as well (Dugan and Green, 1991; Kahn et al., 1986; Bogousslavsky et al., 1985).

Summary and Rationale

The 2VO technique has been used as a model for human disorders which involve chronic cerebrovascular insufficiency. 2VO results in mild, chronic cerebral ischemia which is correlated with progressive cerebral neuropathology and performance deficits on visually-guided behavioural tasks designed to assess learning and memory. It is probable that 2VO also results in retinal ischemia which correlates with retinal neuropathology, loss of pupillary reflex, and impairment of vision. Our laboratory has demonstrated that only a subset of animals subjected to 2VO suffer from pupillary reflex abnormalities, and that only these rats show retinal degeneration and performance deficits on visually-guided tasks. Therefore, it is argued that inferences made about the cognitive effects of 2VO in past studies based on performance on visually-guided tasks may have been confounded by visual dysfunction. Finally, the 2VO technique may provide a useful model for human disorders involving chronic retinal ischemia associated with visual pathology and retinal degeneration such as AD, diabetes, HIV/AIDS, and carotid artery disease.

The purpose of the current study was to investigate (1) the temporal emergence of pupillary reflex dysfunction caused by 2VO, (2) how it relates to the progression of retinal degeneration, as well as (3) the contributory role of light toxicity. The following hypotheses were assessed.

1. Loss of the pupillary reflex results from retinal ischemia. A subset (approximately 2/3)

of Sprague-Dawley 2VO rats suffer retinal ischemia which is sufficient such that the metabolic demands of the retinal photoreceptors are not met, and signal generation and/or transduction of light stimulation is insufficient to elicit the pupillary reflex. These rats suffer eventual degeneration of the retinal ganglion cells and optic nerve.

2. Rats that lose the pupillary reflex subsequently suffer from light toxicity due to their inability to regulate the amount of light which enters the eye. This exacerbates retinal degeneration and in particular leads to the death of retinal ganglion cells and optic nerve degeneration.

As well, to assess the temporal progression of loss of the pupillary reflex and retinal pathology, the study included groups of rats that were sacrificed at 3, 15, and 90 days following 2VO or sham-2VO surgery. The direct and consensual reflex responses of both eyes for all rats were assessed each day for the first week post-surgery, at least once per week thereafter, and immediately before sacrifice.

Assessment of retinal and optic nerve damage was as follows. The eyes of the rats were paraffin embedded, sectioned, and stained as required for the stereological cell counting technique. Cells of the RGCL were counted using stereology. Immunostaining with the Thy-1 antibody which is a selective marker for RGCs (Barnstable and Dräger, 1984; Perry et al., 1984) was also quantified. Thickness measures of the remaining retinal sublayers were calculated as well. Reactive astrocytosis in the optic nerve was assessed by immunohistochemically staining cross sections of optic nerve with a primary antibody to GFAP. Axonal fibre degeneration of the optic nerve was assessed by staining optic nerve cross sections using the Gallyas silver staining technique (Gallyas et al., 1990).

Finally, the potential contribution of light toxicity to retinal degeneration was determined. Half of the animals were housed in complete darkness following surgery until perfusion (dark-rats), while the other half were housed in a regular 12-hour light/dark cycle (light-rats). The dark-rats were only very briefly exposed to light during pupillary reflex observation.

METHODS

Animals

Approximately 4 month old male retired breeder Sprague-Dawley rats (n=74) were obtained from Charles River Laboratories (Montreal, PQ). This strain of rat and particular supplier were chosen due to the fact that loss of pupillary reflex has never been reported by others, nor observed in this laboratory in other strains or in Sprague-Dawley rats from other suppliers (unpublished results). Animals were singly housed in a 12 hour reverse light cycle (on 20:00, off 8:00) with free access to food and water. All procedures were approved by the Animal Care Committee of Carleton University and conformed to the guidelines of the Canadian Council of Animal Care and the ARVO statement for use of animals in ophthalmic and vision research.

Pupillary Reflex

Each animal's pupillary reflexes were examined upon arrival at our laboratory and prior to surgery to verify normal functioning. Direct and consensual responses were assessed in both eyes for all animals in the following manner. Each animal was first allowed to adapt to a dark environment for at least five minutes. One eye was then exposed to a beam of light from an otoscope (Welch Allyn, Skan Falls, NY) to assess the direct reflex response. The otoscope was then immediately directed at the contralateral eye to assess the consensual response. Both eyes were then allowed to readapt to darkness for approximately one minute. The same procedure was then repeated starting with the opposite eye. Pupillary reflexes were examined each day for the first week

following surgery, at least once per week thereafter, and immediately preceding perfusion.

Groups

The experiment was a $2 \times 2 \times 3$ factorial design. The first factor was surgical condition with 2 levels. Animals were randomly assigned to either 2VO surgery or sham-2VO surgery with the condition that the desired group numbers were obtained. The second factor was the post surgical lighting condition with 2 levels. Animals in the dark condition were allowed to recover from surgery in darkness, and post-surgically housed in constant absolute darkness until perfusion. These animals were exposed only very briefly to extremely dim room illumination and a brief direct beam of light from an otoscope during pupillary reflex observation. The rats in the light condition were post-surgically housed in a regular 12 hour reverse light cycle (on 20:00, off 8:00). Room illumination during the light hours was 588 lux measured approximately 75 cm above the floor. The third factor was the post-surgical interval before sacrifice, with 3 levels. Groups of animals were perfused at three different time points, either 3, 15, or 90 days post-surgery. The number of animals in each group is shown in Table 1.

Surgery

Animals were assigned to either 2VO surgery or sham-2VO surgery. 2VO surgery was carried out under ketamine hydrochloride (100 mg/kg i.m.) and methohexital sodium (40 mg/kg i.p.) anaesthesia. Animals were also administered atropine sulphate (0.1 mg i.m.) to prevent respiratory distress during surgery. Both eyes of the animal were covered

	3-day		15-day		90-day	
	Light	Dark	Light	Dark	Light	Dark
2VO	9	9	9	9	9	9
SHAM	2	2	2	2	6	6

Table 1. Number of animals assigned to each surgical, time, and light condition.

with gauze soaked in sterile saline to prevent exposure of the eyes to light during the surgical procedure. A ventral mid-line incision was made, and the common carotid arteries were bilaterally exposed and gently separated from the carotid sheath and vagus nerve in sequence. Each common carotid artery was doubly ligated with 5-0 silk suture at a point approximately 8-10 mm inferior to the origin of the external carotid. The incision was closed and the animal was allowed to recover with free access to food and water. Animals assigned to the sham-2VO surgery underwent the exact same procedure except that the common carotid arteries were not ligated.

Tissue Preparation

At the time of sacrifice, animals were deeply anaesthetized with ketamine hydrochloride and methohexital sodium as described previously. Rats were then transcardially perfused via the left ventricle with 80 ml heparinized saline, followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (fixative). A suture was placed at the top of each eye to facilitate orientation of the eye during paraffin embedding and sectioning. The brains and eyes were then removed and post-fixed in the same fixative for 24 hours at 4°C, then washed in phosphate buffer, dehydrated in a series of graded ethanol solutions, cleared in clearane, and then paraffin embedded. The right eyes of 65 animals were sectioned entirely from bottom to top (transverse) at a thickness of 40 µm on a sliding microtome as required for stereology. All successive sections containing retina were singly mounted on slides dipped in a tissue section adhesive solution (2% Station, Surgipath).

Stereology

In order to visualize cells, retinal sections were stained with hematoxylin. Briefly, sections were de-paraffinized in clearane and rehydrated through a series of graded ethanol solutions and distilled water. Sections were incubated at room temperature in Mayer's hematoxylin (.0083% hematoxylin, .333% ethanol, .15% aluminum ammonium sulphate, .0007% sodium iodate, .0033% citric acid) for 30 minutes. Sections were then placed under running warm water for an additional 30 minutes, then dehydrated, cleared, and coverslipped.

Quantification of the number of neurons within the RGCL was conducted using stereological cell counting techniques by an experimenter blind to group membership. All stereological cell counts were carried out at 1000 $\times$ magnification using a light microscope (Olympus BH-2) connected to a Sony CCD video camera, and a remotely controlled motorized stage system, using commercial stereology software (Stereo Investigator, MicroBrightField, Inc., Colchester, VT). Stereology relies on systematic random sampling during section selection and placement of a sampling grid. An unbiased estimate of the absolute neuron population number within the RGCL was generated in the following manner: First, only cells occurring within a 3-dimensional counting frame were counted. The counting frame was a box with fixed dimensions superimposed on a digitized image of the tissue sample. By focussing to a predetermined start-depth and counting all cells contained within this box while focussing down continuously through focal planes to a fixed end-depth, the counting frame defined a cube of fixed dimensions. A 2-dimensional sampling grid of fixed dimensions was randomly superimposed over the

tissue sample, and counting frames occurred only at each intersection of the grid falling within the structure of interest (RGCL). A predetermined fraction of all sections was quantified in this manner at a fixed interval, with the first slide being randomly selected within the first interval. Therefore, all cells occurring within a known fraction of the entire structure of interest were counted. From this, an unbiased estimate of the total number of neurons within the structure was calculated. A brief pilot study using practice tissue was performed to determine an appropriate section sampling interval (every ninth section), sampling grid size ($120\mu\text{m} \times 120\mu\text{m}$), and counting frame size ($18\mu\text{m} \times 18\mu\text{m} \times 10\mu\text{m}$), such that an acceptable coefficient of error (CE Gundersen = 0.08) associated with the population estimate was obtained (for review see West, 1999; Gundersen and Jensen, 1987). The start-depth of the counting frame was 3 μm below the surface of the tissue, providing a guard-zone to prevent counting of lost caps and neurons damaged during sectioning.

Thy-1

In order to determine if reductions of neuron numbers in the RGCL reflected loss of RGCs specifically, sections were immunohistochemically stained with a monoclonal antibody raised in mouse against Thy-1 (PharMingen International). Thy-1 expression within the retina is specific to RGC bodies and their processes (Barnstable and Dräger, 1984; Perry et al., 1984). Thy-1 immunostaining was performed only on retinas from the 90-day group because a significant reduction of neurons in the RGCL had occurred by 90 days in eyes which lacked the pupillary reflex.

Sections (3 per eye) adjacent to those selected for thickness measures (see below) were used. Briefly, sections were deparaffinized, rehydrated, and washed in 10 mM phosphate buffered saline (PBS), pH 7.2. Sections were incubated overnight in mouse anti-Thy-1 (1:1000, PharMingen International) diluted in a blocking buffer of .3% lambda carrageenan, .3% bovine serum albumin, and .3% triton X-100 in PBS. Lambda carrageenan and bovine serum albumin block non-specific binding of the antibody. Triton X-100 is a surfactant used to recover membrane components under non-denaturing conditions. Sections were then washed in PBS before being incubated for 3 hours in a secondary antibody, biotinylated anti-mouse Ig (1:100, Amersham). Following another wash in PBS, sections were incubated for 3 hours in a tertiary antibody; streptavidin biotinylated horseradish peroxidase complex (1:100, Amersham). Sections were washed in PBS, then presoaked in .02% 3,3'-diaminobenzidine in 50 mM Tris buffer (0.76% trizma pre-set crystals), pH 7.4 (DAB solution). After 32 μ l of 30% hydrogen peroxide was added to the DAB solution, sections were allowed to incubate for another 7 minutes. This step was repeated again with the addition of another 32 μ l of 30% hydrogen peroxide and a 7 minute incubation. Finally, the sections were dehydrated, cleared, and coverslipped.

Thy-1 IR was quantified using commercial imaging software (MCID, Imaging Research, St. Catharines, ON) at 200 $\times$ magnification with the same microscope and camera previously described. Multiple measures (approximately 15-20) were conducted on each section by determining the proportion of area which showed Thy-1 IR. This was carried out on a sampling area containing an approximately constant length of retina.

Measures were averaged across sections for each eye. All Thy-1 IR quantification was conducted by an experimenter blind to group membership.

Retinal Sublayers

The thicknesses of other retinal sublayers (ONL, OPL, INL, IPL) were measured by an experimenter blind to group membership as well. Thickness measures were carried out at 400× magnification with the previously described microscope and camera and MCID software. Three or 4 hematoxylin stained sections containing the central retina were selected for each eye. The sections chosen were adjacent to ones that had been used for stereology. Multiple thickness measures (approximately 120-160 measures per section) were averaged for each layer on each section and these were then averaged across sections to yield the thickness of the 4 layers for each eye.

Optic Nerve

To assess fibre degeneration and reactive astrocytosis in the optic nerve, cross sections of the nerve were stained with Gallyas silver (Gallyas et al., 1990) and with an antibody to GFAP (Sigma) respectively.

One section containing a cross section of optic nerve was selected from each eye for Gallyas staining and quantification. Briefly, sections were deparaffinized in clearane, and washed twice for 10 minutes in 100% 1-propanol. Sections were then incubated overnight in 100% 1-propanol containing .8% distilled water and 1.2% concentrated sulfuric acid at 56°C. The sections were then rehydrated and reacted with .5% glacial

acetic acid for 10 minutes. Next, the sections were reacted with developer (100 ml 10% sodium carbonate, 90 ml distilled water, .25 g ammonium nitrate, .2 g silver nitrate, 10 ml 29% tungstosilic acid, .5 ml 37% formaldehyde, .25% glacial acetic acid) for 10 minutes. Finally, a 30-minute wash in 1% glacial acetic acid, then a 5-minute wash in propanol were followed by immersion in anise oil for 5 minutes prior to coverslipping.

Sections adjacent to those selected for Gallyas staining were used for GFAP IR. Briefly, sections were processed exactly as described previously for Thy-1 IR with the following exceptions. The primary antibody used was mouse anti-GFAP (1:600, Sigma). The DAB solution contained 1.2% ammonium nickel sulphate. As well, sections were counterstained following the DAB reaction with 3.4% pyronin Y in .05 N acetate buffer, pH 4.8, prior to dehydration, clearing, and coverslipping.

Quantification of Gallyas staining and GFAP IR was conducted using MCID. The proportional area of the optic nerve stained was calculated by an experimenter blind to group membership.

RESULTS

Surgery and Pupillary Reflex

Of the 74 animals that underwent surgery (20 sham; 54 2VO), 1 animal from the 2VO/3-day/light group died. Of the 53 remaining 2VO animals, 18 animals (34.0%) eventually lost the direct and consensual pupillary reflex to light in both eyes. Loss of pupillary reflex was defined as failure of the pupil to constrict following a minimum 10 second exposure to a direct, concentrated beam of light from the otoscope. Another 13 animals (24.5%) eventually suffered unilateral loss of pupillary reflex. Unilateral loss of reflex was characterised by a lack of direct response in one eye (afflicted eye), accompanied by a lack of consensual response in the contralateral eye. However, in all cases of unilateral reflex loss, the afflicted eye retained the consensual response to light stimulation of the contralateral eye. In some cases, animals that eventually suffered bilateral loss of the reflex initially displayed unilateral loss of reflex, followed by loss in the second eye. Loss of pupillary reflex often occurred immediately following surgery, most often within 3 days, and in all cases within 7 days post-surgery. Beyond post-surgical day 7, the state of pupillary reflex function remained constant for all animals. The remaining 2VO animals (n=22), as well as all sham animals (n=20), displayed no pupillary reflex abnormalities. These results are shown in Table 2.

Figure 4 shows that the loss of pupillary reflex (unilateral or bilateral) was not determined by the light vs. dark housing of the animal. The proportion of 2VO animals suffering from loss of normal pupillary reflex overall was 31/53 (58.5%) as follows. 14/26 (53.8%) in the dark condition and 17/27 (63.0%) in the light condition. Loss of pupillary

		3-day		15-day		90-day	
	Reflex	Light	Dark	Light	Dark	Light	Dark
2VO	intact	3	4	6	3	3	3
	unilateral	2	2	1	4	1	3
	bilateral	3	3	2	2	5	3
SHAM	intact	2	2	2	2	6	6

Table 2. Number of animals with intact pupillary reflex, unilateral loss, and bilateral loss in each condition by 7 days following surgery.

Figure 4: Number of 2VO animals with no loss, unilateral loss, or bilateral loss of pupillary reflex. There were no significant effects of day or light condition.

reflex occurred randomly across groups in 2VO animals as indicated by a nonparametric Chi-square test ($\chi^2=2.30$, $df=2$, $p=.316$).

For statistical analyses of the histological data, the eyes could be classified according to group (sham vs 2VO), whether the eye showed the reflex (flex) or not (noflex), post-surgical interval (3, 15 or 90 days) and post-surgical housing condition (light vs. dark). Since all of the shams retained the reflex, there were 3 main groups (Sham, 2VO-flex, and 2VO-noflex) that could be further subdivided. Group sizes are shown in Table 3. Subsequent analyses usually consisted of an initial $3 \times 3 \times 2$ factorial design with group (sham, 2VO-flex, and 2VO-noflex), post-surgical interval (3-day, 15-day, and 90-day), and post-surgical lighting condition (light, dark) as the between subjects variables. Since the eyes from the 3-, 15- and 90-day, light and dark housed sham groups showed no differences on any measure, their scores could be collapsed into a single group (CON) for statistical comparisons. Data for the sham animals which comprised the CON group are shown in Appendix B.

Retinal Ganglion Cell Layer

Stereology

An estimate of the total number of neurons within the RGCL was generated using stereological counting techniques. Using systematic random sampling, every ninth section was stained with hematoxylin and enumerated. Images of these sections are shown in Figure 5. Two 2VO-noflex eyes (1 from the 15-day/dark group and 1 from the 90-day/light group) were found to have severely degenerated retinas. The estimate for

	3-day		15-day		90-day	
	Light	Dark	Light	Dark	Light	Dark
2VO-flex	6	4	6	5	5	4
2VO-noflex	3	5	3	4	3	5
SHAM	2	2	2	2	2	2

Table 3. Number of eyes used for histological analyses in each group (N=65).

Figure 5: Digital images of hematoxylin stained sections of retina for CON (A, B), 2VO-flex (C, D), and 2VO-noflex (E, F) at 90 days in the light condition (A, C, E) and dark condition (B, D, F). Note that all CON and 2VO-flex retinas in both light and dark conditions look similar (A, B, C, and D). In the 2VO-noflex/dark retina (F), note thinned or missing OPL (←), thinned IPL (◀), and fewer RGCL nuclei (◀◀) relative to the CON and 2VO-flex retinas. In the 2VO/noflex/light retina, note the absence of an ONL (▶), thinned or missing OPL (→), thinned IPL(▶), and fewer nuclei in the RGCL (→) relative to the CON and 2VO-flex retinas. Scale bar represents 50μm.

— 50 μ m

neuron population in these retinas was 0 as no discernable RGCL existed within these eyes. Three eyes were omitted from this analysis (1 2VO-flex/90-day/dark, 1 sham/15-day/light, and 1 2VO-noflex/15-day/dark) because accurate counts could not be conducted due to tissue damage occurring during processing.

A $3 \times 3 \times 2$ factorial ANOVA with group, day, and light as between subjects variables (Figure 6) indicated a significant main effect of group ($F(2, 44)=18.18, p<.001$). Scheffé's test showed that the 2VO-noflex group had fewer neurons within the RGCL ($p<.001$) as compared to sham and 2VO-flex eyes which did not differ. There was also a significant main effect of day ($F(2, 44)=4.70, p=.014$), due to a reduction of RGCL neurons within the 15-day and 90-day groups (Scheffé's test, $p=.045, p=.012$ respectively) compared to the 3-day group. No significant interactions or effects of light were found.

A priori ANOVAs were conducted for 2VO-flex, 2VO-noflex, and CON subgroups at the three different time points using the Bonferroni correction for non-orthogonal comparisons where applicable. The 2VO-noflex eyes showed significantly fewer RGCL neurons compared to CON eyes by 15 days ($p<.05$). By 90 days an even more severe reduction was apparent, with a significant loss of cells as compared to both CON ($p<.001$) and 2VO-flex eyes ($p<.001$). The results are shown in Figure 7.

Thy-1

Figure 8 shows images of sections stained for Thy-1 IR. The percent RGCL area that showed Thy-1 IR was quantified. All 2VO-noflex eyes failed to show Thy-1 IR and were assigned a value of 0 by the experimenter (blind to group membership). All sham

Figure 6: Stereological population estimates of neurons in the RGCL, all subgroups.

Y=flex, N=noflex. Error bars represent S.E.M.

Figure 7: Stereological population estimates of neurons in the RGCL. There was a significant main effect of group ($F(2, 44)=18.18$, $p<.001$). 2VO-noflex eyes had less cells in the RGCL (Scheffé's, $p<.001$) than the sham and 2VO-flex groups. There was a significant main effect of day ($F(2, 44)=4.70$, $p=.014$), with a reduction at 15 (Scheffé's test, $p=.045$) and 90 (Scheffé's test, $p=.012$) days compared to 3-day eyes. 2VO-noflex eyes had significantly less RGCL neurons than CON at 15 days (Bonferonni, $p<.05$), and less than CON ($p<.001$) and 2VO-flex eyes at 90 days ($p<.001$). * and ** represent difference from CON at the $p<.05$ and $p<.01$ levels respectively; †† represents difference from the 2VO-flex counterpart at the $p<.01$ level. Y=flex, N=noflex. Error bars represent S.E.M.

Figure 8: Digital images of sections immunohistochemically stained for Thy-1 from 90-day CON (A), 2VO-flex(B), and 2VO-noflex (C) eyes. Note the absence of Thy-1 immunoreactivity ( ) in the IPL and RGCL of the 2VO-noflex retina relative to the CON and 2VO-flex retinas. Scale bar represents 50 μ m.

— 50 μ m

and 2VO-flex eyes showed some degree of Thy-1 IR. A 3×2 factorial ANOVA with group and light as between subjects variables (Figure 9) indicated a significant main effect of group ($F(2, 16)=16.82, p<.001$). No effect of light or group by light interaction was found. The 2VO-noflex eyes differed from the sham and 2VO-flex groups (Scheffé's test, $p=.014$, and $p<.001$ respectively) as shown in Figure 10.

Retinal Sublayers

Outer Nuclear Layer

Figure 11 summarizes the data for the ONL. A $3 \times 3 \times 2$ factorial ANOVA with group, day, and light as between subjects variables indicated a significant effect of day ($F(2, 47)=16.97, p<.001$), a significant group by day interaction ($F(4, 47)=3.95, p=.008$), a significant day by light interaction ($F(2, 47)=8.50, p=.001$), and a significant three-way group by day by light interaction ($F(4, 47)=3.17, p=.022$). *Post hoc* (Scheffé's test) comparisons revealed that only the 90-day 2VO-noflex eyes from the light condition showed thinning of the ONL. This thinning was significant as compared to CON eyes ($p=.004$) and to the 90 day 2VO-flex eyes ($p=.029$) from the light condition. As well, their thickness was less than that of the 90-day 2VO-noflex eyes in the dark condition ($p=.027$) which were not different from CON. Hence, post-surgical housing in the light was responsible for the reduction of ONL thickness in the 90 day 2VO noflex eyes.

These results are obvious in Figure 5.

Figure 9: Percent area of retina showing Thy-1 immunoreactivity at 90 days, all subgroups shown. L=light, D=dark. Error bars represent S.E.M.

Figure 10: Percent area of retina showing Thy-1 immunoreactivity at 90 days. There was a main effect of group ($F(2, 16)=16.82, p<.001$). 2VO-noflex eyes differed from the sham and 2VO-flex groups (Scheffé's test, $p=.014$, and $p<.001$ respectively). * represents difference from CON at the $p<.05$ level; †† represents difference from 2VO-noflex counterpart at the $p<.01$ level. Error bars represent S.E.M.

Figure 11: Layer thickness measures of the outer nuclear layer of the retina. There was a significant main effect of day ($F(2, 47)=16.97, p<.001$), a group by day interaction ($F(4, 47)=3.95, p=.008$), a day by light interaction ($F(2, 47)=8.50, p=.001$), and a three-way group by day by light interaction ($F(4, 47)=3.17, p=.022$). Only the 90-day/2VO-noflex/light eyes showed reduced layer thickness compared to CON ($p=.004$), the 2VO-flex counterpart ($p=.029$) and the 2VO-noflex/dark counterpart ($p=.027$). ** represents difference from CON at the $p<.01$ level; † represents difference from 2VO-flex counterpart at the $p<.05$ level; ‡ represents difference from 2VO-noflex light counterpart at the $p<.05$ level. Y=flex, N=nox. Error bars represent S.E.M.

Outer Plexiform Layer

ANOVA of the OPL thickness data revealed a significant main effect of group ($F(2, 47)=12.07, p<.001$), day ($F(2, 47)=26.29, p<.001$), and a group by day interaction ($F(4, 47)=4.84, p=.002$). No main effects or interactions with light were found. As shown in Figure 13, the 90-day 2VO-noflex group showed significantly reduced OPL thickness compared to CON ($p<.001$) and 2VO-flex ($p=.001$) groups. The 2VO-noflex, 90 day eyes from both the dark and the light conditions at 90 days showed OPL thinning as shown in Figure 12.

Inner Nuclear layer

The data for the INL are shown in Figure 14. The overall $3 \times 3 \times 2$ ANOVA revealed a significant main effect of day ($F(2, 47)=4.54, p=.016$) and a day by light interaction ($F(2, 47)=3.39, p=.042$). The 90-day group showed thinning of the INL relative to the 3-day group (Scheffé's test, $p=.012$). No main effects or interactions involving group were found. Further, no groups differed from CON eyes at any time point in any condition. Finally no two groups differed among all multiple comparisons.

Inner Plexiform Layer

The data for IPL thickness are shown in Figure 15. Significant main effects of group ($F(2, 47)=8.59, p=.001$) and day ($F(2, 47)=5.67, p=.006$) were found as well as a significant group by day interaction ($F(4, 47)=2.57, p=.05$). As shown in Figure 16, Scheffé's test showed that the IPL thickness of the 2VO-noflex eyes was less than that of

Figure 12: Thickness measures of the outer plexiform layer of the retina. At 90 days, the 2VO-noflex eyes showed reduced OPL layer thickness in both the light ($p<.001$) and dark ($p=.014$) conditions. Only the 2VO-noflex eyes in the light differed from the 2VO-flex counterpart at 90 days ($p=.025$). * and ** represent difference from CON at the $p<.05$ and $p<.01$ levels respectively. † represents difference from the 2VO-flex counterpart at the $p<.05$ level of significance. Y=flex, N=no flex. Error bars represent S.E.M.

Figure 13: Layer thickness measures of the outer plexiform layer of the retina. There was a significant main effect of group ($F(2, 47)=12.07, p<.001$), day ($F(2, 47)=26.29, p<.001$), and a group by day interaction ($F(4, 47)=4.84, p=.002$). The 2VO-noflex group showed significantly reduced layer thickness compared to CON ($p<.001$) and the 2VO-flex counterpart ($p<.001$) at 90 days. ** represents difference from CON at the $p<.01$ level; †† represents difference from 2VO-noflex counterpart at the $p<.01$ level. Y=flex, N=noflex. Error bars represent S.E.M.

Figure 14: Layer thickness of the inner nuclear layer of the retina. There was a significant effect of day ($F(2, 47)=4.54$, $p=.016$) and a day by light interaction ($F(2, 47)=3.39$, $p=.042$) where the 90-day group showed reduced layer thickness overall compared to the 3-day group (Scheffé's test, $p=.02$). No two groups differed by multiple comparison analyses. Y=flex, N=noflex. Error bars represent S.E.M.

Figure 15: Layer thickness measures for the inner plexiform layer of the retina, all subgroups shown. Y=flex, N=no flex. Error bars represent S.E.M.

Figure 16: Layer thickness measures for the inner plexiform layer of the retina. There was a significant main effect of group ($F(2, 47)=8.59, p=.001$), day ($F(2, 47)=5.67, p=.006$), and a group by day interaction ($F(4, 47)=2.57, p=.05$). The 2VO-noflex eyes differed from CON eyes ($p=.011$) and the 2VO-flex eyes ($p=.019$) at 90 days. * represents difference from CON at the $p<.05$ level of significance; † represents difference from the 2VO-flex counterpart at the $p<.05$ level of significance. Y=flex, N=no flex. Error bars represent S.E.M.

both the CON eyes ($p=.011$) and the 2VO-flex eyes ($p=.019$) at the 90-day time point.

No significant contribution of light was found by these analyses.

Optic Nerve

Gallyas Silver Stain

For 2 cases, the optic nerve appeared to have degenerated to obliteration. Therefore, 2 eyes from the 90-day/2VO-noflex/light group were omitted from analysis as it was not possible to quantify staining in a structure which was virtually missing. Visual observation revealed that in general, the optic nerves of the 2VO-noflex eyes were substantially shrunken as compared to the 2VO-flex and CON eyes, as shown in Figure 17. Gallyas staining was not apparent in any of the CON eyes and it was apparent, but very faintly, in only 2 of the 27 2VO-flex eyes. The remaining 24 2VO-noflex eyes all showed substantial staining. Atrophy and staining of optic nerves of the 2VO-noflex animals was apparent by 3 days and at the 15- and 90-day time points as well. The 3-way ANOVA revealed a significant group difference ($F(2, 45)=115.45$, $p<.001$) with the 2VO-noflex group showing significantly increased staining over the CON ($p<.001$) and 2VO-flex ($p<.001$) groups which did not, as shown in Figure 19. No significant effects of day or light were found (Figure 18).

GFAP Immunoreactivity

Two eyes were omitted from analysis; one section suffered tissue damage during processing (2VO-flex/15-day/dark) and one section contained an optic nerve which was

Figure 17: Digital images of optic nerve cross sections of CON (A, B), 2VO-flex (C, D), and 2VO-noflex (E, F) eyes at 90 days stained with Gallyas silver (A, C, E), and immunohistochemically with monoclonal anti-GFAP antibody (B, D, F). Note shrinkage of the optic nerve in the 2VO-noflex (E and F) condition relative to the CON and 2VO-flex conditions (A, B, C, D). Note the absence of positive silver staining in the CON (A) and 2VO-flex (C), and abundant positive silver staining in the 2VO-noflex optic nerve (E). Note increased GFAP IR in the 2VO-noflex optic nerve (F) as compared to the CON (B) and 2VO-flex (D) conditions. Scale bar represents 500 μm .

Figure 18: Percent area of optic nerve stained with Gallyas silver, all subgroups shown.

* and ** represent difference from CON at the $p<.05$ and $p<.01$ levels of significance respectively; † and †† represent difference from 2VO-flex at the $p<.05$ and $p<.05$ levels of significance respectively. Y=flex, N=noflex. Error bars represent S.E.M.

Figure 19: Percent area of optic nerve stained with Gallyas silver. There was a main effect of group ($F(2, 45)=115.45$, $p<.001$). The 2VO-noflex eyes displayed a higher proportion of positively stained optic nerve than both the CON ($p<.001$) and 2VO-flex eyes ($p<.001$). ** represents difference from CON at the $p<.01$ level of significance; †† represents difference from 2VO-flex at the $p<.01$ level of significance. Y=flex, N=no flex. Error bars represent S.E.M.

Gallyas Silver Stain in Optic Nerve

degenerated to a degree where it could not be analysed (2VO-noflex/90-day/light). As was apparent for the Gallyas silver staining, the GFAP stained sections revealed that the optic nerves of the 2VO-noflex eyes were indeed shrunken. However, sections from all eyes except one (2VO-flex/90-day/dark) showed positive staining for GFAP IR as shown in Figure 17. No significant main effects were found for group, day, or light (Figure 20). The group by day interaction just missed significance ($F(4, 45)=2.47, p=.058$). As shown in Figure 21, overall the 2VO-noflex group had the lowest GFAP IR at 3 days and the highest GFAP IR at 90 days.

Figure 20: Percent area of optic nerve showing GFAP immunoreactivity, all subgroups shown. Y=flex, N=noflex. Error bars represent S.E.M.

Figure 21: Percent area of optic nerve showing GFAP immunoreactivity. Y=flex, N=noflex. Error bars represent S.E.M.

DISCUSSION

Of the animals that underwent 2VO surgery in this study, approximately 58.5% suffered loss of the pupillary reflex in one or both eyes in a very consistent pattern. As well, these animals invariably showed visual system pathology relative to the 2VO-flex and sham animals. The general findings of this study were that the afflicted eyes of 2VO-noflex animals showed progressive retinal and optic nerve degeneration beginning as early as 3 days post-ligation and worsening over time. The 2VO-noflex eyes were shown to have fewer neurons and a lack of Thy-1 IR in the RGCL, thinner inner and outer plexiform layers, and degenerated optic nerves whether post-surgically housed in light or dark conditions. Further, the 2VO-noflex eyes eventually showed a severe loss of photoreceptors as indicated by a thinner or absent ONL when housed in the light condition only. It is therefore argued that ischemia directly caused loss of pupillary reflex and the initial retinal and optic nerve pathology, and that subsequent light toxicity was responsible for the loss of photoreceptors.

Pupillary Reflex

Loss of the pupillary reflex followed a consistent pattern. First, this loss was often evident immediately following surgery. The anaesthesia used during surgery had the effect of dilating both pupils of all animals. For some animals, one or both pupils remained dilated following surgery and thereafter. All sham animals and most 2VO animals regained the pupillary reflex shortly following surgery (2-3 hours). Any further change in pupillary function always occurred within 7 days following surgery. 2VO-noflex animals

often suffered unilateral loss of reflex. In 13 of 31 (~42%) 2VO-noflex animals, unilateral loss was permanent. Some 2VO-noflex animals that initially presented with unilateral dysfunction eventually suffered bilateral loss of the reflex.

Whether transient or permanent, the following conditions applied for all cases of unilateral reflex loss. Exposure of the afflicted eye to a light stimulus elicited no direct reflex, nor a consensual reflex in the contralateral eye. Exposure of the contralateral (unafflicted) eye to a light stimulus elicited the appropriate direct response in that eye, accompanied by the consensual response in the afflicted eye. In cases of bilateral dysfunction, no direct or consensual responses were ever elicited by the light stimulus.

It is possible to determine from these observations where the breakdown of the pupillary reflex pathway occurred for all cases of unilateral loss. Initiation of the reflex signal originates in the retina, and leaves the eye via the optic nerve which projects bilaterally to the olivary pretectal nuclei (OPN) (see Figure 3). From there the signal is projected bilaterally to the Edinger-Westphal nuclei. Thus, the signal crosses at two points before it continues through the efferent branch of the pathway, which is unilateral for each eye. In the case of unilateral dysfunction, the efferent branch (motor pathway) of the afflicted eye was fully functional as demonstrated by spared pupillary constriction of the consensual response. Therefore, failure must have occurred within the afferent branch of the pathway.

It can also be inferred that both the efferent and afferent branches of the contralateral (unafflicted) eye were intact, as light stimulation elicited both a direct response from it as well as a consensual response in the afflicted eye. Furthermore, it can

be concluded that failure within the afferent branch of the afflicted eye occurred at some point before any bilateral projection (crossover) point. If the reflex signal had been successfully generated and transduced beyond the optic nerve, both direct and consensual responses would have been elicited due to signal crossover and intact motor pathways.

Therefore, it can be concluded that unilateral reflex dysfunction was caused by failure of the retina and/or optic nerve to generate and/or propagate the pupillary reflex signal. It seems most likely that in the case of bilateral loss, both eyes suffered the same pathology as in the case of unilateral loss. The histological analyses of the retina support this interpretation of the reflex data.

RGCL Pathology

RGCL pathology was assessed by estimating the total number of neurons within the RGCL using stereology, as well as by quantifying Thy-1 IR, a specific marker for RGCs. The number of cells in the RGCL of 2VO-noflex eyes was significantly reduced after 15 days of 2VO relative to the CON eyes. This reduction was more severe after 90 days when there were significantly fewer cells relative to both the CON and 2VO-flex groups.

Thy-1 IR was absent in all 2VO-noflex eyes at the 90-day time point. Both the CON and 2VO-flex eyes showed a considerable amount of Thy-1 IR and did not differ from one another. It is therefore concluded that the RGC population was virtually eliminated in the 2VO-noflex eyes by 90 days.

Aside from the two retinas which were virtually obliterated and showed no RGCL,

2VO-noflex eyes demonstrated a considerable number of remaining cells in the RGCL, despite the apparent elimination of RGCs. It is likely that these were displaced amacrine cells. However, it cannot be ruled out that some were RGCs. It may be possible that expression of Thy-1 antigen, which is a glycoprotein found on the surface of the cell, was absent only if the RGCs were at an advanced stage of degeneration.

These results suggest a pathological sensitivity of the RGCs to 2VO which remains to be explained. A recent study from our laboratory demonstrated that progressive apoptotic cell death beginning as early as 10 weeks after 2VO, occurs in a subpopulation of RGCs which express platelet activating factor receptor mRNA (unpublished results). However, morphological evaluation combined with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) did not indicate apoptotic cell death following 2 weeks of 2VO, whereas the current findings showed reduced cell counts by 15 days in 2VO-noflex eyes. It is therefore suggested that a significant degree of necrotic cell death must have occurred by this time point.

One possible explanation for selective RGC death is that 2VO rapidly damaged the optic nerve, which consists of the axons of the RGCs. Subsequent retrograde degeneration may then have been the cause of RGC loss. In support of this, optic nerve fibre degeneration was evident here as early as 3 days following 2VO, as demonstrated with the Gallyas silver staining technique. Others have also noted that the optic nerve suffers substantial degeneration within the first week following 2VO (Ohta et al., 1997; Wakita et al., 1995).

Evidence suggests that cerebral white matter is highly vulnerable to ischemia

(Pantoni et al., 1996) and ischemic damage to optic nerve axons has been demonstrated with *in vitro* models (Stys et al., 1992; Stys, 1998). Hence, it is feasible that one of the first consequences of 2VO is optic nerve damage. Future studies should address this possibility and why it occurs in a sub-population of rats subjected to 2VO.

The findings for the RGCL are noteworthy in that to our knowledge, this is the earliest time point at which a significant reduction of the cell population within the RGCL has been detected following 2VO. These results disagree with the conclusions of a very recent study (Osborne et al., 1999) which found no abnormalities of retinal morphology after 90 days of 2VO, aside from a general thinning of the retina which was unquantifiable. Further, by 9 months, only thinning of the ONL was observed, along with general thinning of the Thy-1 IR layer which was also unquantifiable. A number of differences may account for the discrepancies. First, this study of Osborne et al. did not include assessment of the pupillary reflex, so it is possible that the eyes that they examined were at least in part from rats with an intact reflex, and presumably these eyes suffered less ischemic damage. Second, more accurate, stereology-based, neuron counting techniques were employed in the current study and this may have increased the power to detect differences in neuron numbers. The technique is unbiased in that no assumptions are made concerning the size or orientation of neurons. Therefore, counts remain reliable and accurate despite potential tissue shrinkage.

Retinal Sublayer Pathology

Pathology of retinal sublayers was assessed by measuring the average thickness of

layers on sections stained with hematoxylin. The ONL, OPL, INL, and IPL thicknesses were quantified. No significant differences between groups were noted for any of the retinal sublayers until the 90-day time point following surgery. By 90 days post-surgery, the 2VO-noflex eyes demonstrated thinning of the OPL and IPL. The ONL was thinner or missing in 2VO-noflex eyes in the light condition only.

Outer Nuclear Layer

The ONL contains the cell bodies of the photoreceptors. Severe loss of photoreceptor cells, as demonstrated by a thin or missing ONL, occurred here much later than the loss of pupillary reflex. As well, only 2VO-noflex eyes in the light condition showed photoreceptor loss. However, the loss of the pupillary reflex occurred equally in the light and dark conditions. Therefore it is unlikely that functional failure of the photoreceptors caused the loss of the reflex, as was initially hypothesised. As well, it is unlikely that the photoreceptive neurons were especially vulnerable to ischemic damage since the ONL appeared histologically intact in 2VO-noflex eyes from the dark condition. It is therefore concluded that the destruction of photoreceptors was subsequent to reflex loss, and was mediated by light toxicity resulting from the inability of 2VO-noflex eyes to moderated the amount of retinal exposure to light.

Outer Plexiform Layer

The OPL is the site of interconnection between the photoreceptors, and the horizontal and bipolar cells. This layer proved to be thinner in 2VO-noflex eyes after 90

days in both the light and dark conditions. Therefore pathology within this layer was to some extent caused by ischemia. However, while the 2VO-noflex groups in the light and dark conditions did not show a statistically significant difference, the eyes from the light condition showed the most substantial thinning relative to controls, suffering near elimination of the OPL. Thus the OPL fell victim to a combination of ischemic and phototoxic insult.

Inner Nuclear Layer

The INL contains the horizontal, bipolar, and majority of amacrine cell bodies. The INL data is somewhat ambiguous and difficult to interpret. No significant differences were observed between the CON, 2VO-flex, or 2VO-noflex eyes. The only significant finding was that in general, the eyes showed slight thinning of the INL at the 90-day time point relative to 3 days post-surgery. A significant day by light interaction was ambiguous as well, as no explicable trend was apparent. Rather, the effect of light seemed to be opposite at 15 and 90 days post-2VO. This effect may be attributable to the fact that there was one 2VO-noflex eye with complete elimination of the retina in both the 15-day/dark and 90-day/light conditions. Finally, because no differences were found between the 2VO-flex, 2VO-noflex and CON groups, it is concluded that no clear effect of ischemia or light toxicity consistently damaged the INL. Hence, these inner retinal neurons seem minimally affected by ischemia and phototoxicity.

Inner Plexiform Layer

The IPL is the site of interconnection between the bipolar and amacrine cells of the INL, and the RGCs. This layer was thinner in 2VO-noflex eyes than in both the CON and 2VO-flex eyes after 90 days. Because no effect of light condition was found, it is likely that degeneration within this layer was mediated by ischemic damage. It is significant to note that thinning of the IPL was subsequent to degeneration of the RGCL. It is most likely that thinning of the IPL reflected degeneration of the RGC dendrites as opposed to degeneration of the processes of the INL neurons. This can be inferred from the fact that the 2VO-noflex eyes suffered loss of RGCs but no apparent loss of INL cells. As well, Thy-1 IR was absent within the IPL in all 2VO-noflex eyes by 90 days (see above), indicating an elimination of RGC processes within this layer.

Optic Nerve Pathology

The optic nerve consists of the axonal fibre bundles of the RGCs, and becomes the optic tract as it enters the brain. Cross sections of optic nerve were stained with the Gallyas silver technique, and for GFAP IR to quantify degenerating fibres and reactive astrocytosis respectively.

Gallyas Silver

Degeneration of the optic nerve was apparent by 3 days post-surgery in the 2VO-noflex eyes as shown by substantial Gallyas silver staining. All 2VO-noflex eyes were positively stained. Conversely, all 2VO-flex and CON eyes, with the exception of two

barely stained 2VO-flex eyes, showed no staining at all. This indicated that the optic nerve had sustained damage by the earliest time point assessed in the 2VO-noflex group. Because there was no difference between the light and dark conditions, it is inferred that this damage was a result of insufficient blood flow to the optic nerve. No differences were observed over time. This is not a surprising finding, as the Gallyas technique only results in positive staining within 3 days of injury (Gallyas et al., 1992). Therefore, unless the rate of degeneration changed considerably between time points, changes in the degree of staining would not be expected. This indicates that optic nerve degeneration was still occurring at a substantial rate after 90 days of 2VO in the eyes that lacked the reflex.

Degeneration of the optic nerve was the only observation which temporally coincided with loss of pupillary reflex. It is therefore concluded that loss of pupillary reflex in 2VO-noflex eyes was caused by failure of the optic nerve to transduce light signals from the retina, as a result of ischemic insult.

GFAP immunoreactivity

Cross sections of optic nerve immunohistochemically stained for GFAP IR did not show any significant differences among groups. This observation was perplexing, as other studies have observed increased GFAP in the optic nerve (Wakita et al., 1995) and retina (Barnett and Osborne, 1995) as early as 7 days following 2VO. Although not statistically significant, GFAP IR was lowest among all groups in the 2VO-noflex group at 3 days, and highest in the 2VO-noflex group at 90 days. This defined an apparent trend characterised by an initial drop followed by an increase relative to CON eyes. This trend has been

observed previously in a study using a model of elevated intraocular pressure (IOP) (Johnson et al., 2000). It was shown that GFAP IR was reduced following 7 days of elevated IOP. Beyond 14 days of elevated IOP, GFAP IR increased to normal levels. It is possible that 2VO-noflex eyes, which seem to suffer more severe ischemic insult than 2VO-flex eyes, react in a manner consistent with models of elevated IOP. Reduced GFAP IR has been attributed to cytoskeletal reorganization following loss of gap junctions and cellular proliferation (Johnson et al., 2000).

Suggestions for Future Research

This study marks the third experiment from this laboratory which has demonstrated loss of pupillary reflex in a majority of 2VO rats. To our knowledge, loss of pupillary reflex has never been reported by any other group using the 2VO model, despite reports of visual system pathology. In our laboratory, the loss of pupillary reflex after 2VO has only been observed in Sprague-Dawley rats from Charles River Laboratories (Montreal). Experiments with Fischer rats, and Sprague-Dawley rats from an alternate supplier (Harlan, Indianapolis, IN), have not resulted in loss of pupillary reflex (unpublished results). Therefore, this particular strain of rat is more vulnerable to the effects of retinal ischemia. Further, there appeared to be 2 distinct subgroups of this strain of rat: (1) those that suffered loss of pupillary reflex accompanied by retinal and optic nerve degeneration, and (2) those that did not.

It has yet to be determined why only a subset of these rats lose the reflex and suffer visual system pathology. One explanation would be that this subset suffers more

severe reduction of RBF following 2VO. This could be the result of vascular differences rendering some rats unable to tolerate ligation of the common carotid arteries. It has been noted that the structure of the circle of Willis, which may be responsible for retrograde supply of blood to the retina during 2VO (Block et al., 1992), varies considerably among rats (Scremin, 1995). A larger diameter circle of Willis or larger posterior arteries in some animals would allow for reverse perfusion of the internal carotid and pterygopalatine arteries and thence the retina (see Figure 1) in some animals but not others. The latter rats may lose their pupillary reflex as a result of the greater optic nerve ischemia that they experience.

Measurement of CBF and RBF should be undertaken to examine for differences in retinal (and cerebral) ischemia between 2VO-noflex and 2VO-flex groups. Suggested techniques for such measurement include laser doppler blood flow quantification systems, and dynamic magnetic resonance imaging (MRI) of cerebral and retinal blood flow.

Alternatively, differences may be attributable to characteristics of the retina. It is known that albino rats have a reduced contralateral reflex pathway, and they have more variable reflex responses in general than pigmented rats (Chan et al., 1995). It has also been demonstrated that the retinas of albino rats are more susceptible to phototoxic insult (Humpel et al., 1992) and ischemic damage (Rukhsana and Osborne, 2000). The suggested explanation for resilience of the pigmented strains was that melanin within pigmented cells may function as an antioxidant protecting the cells from oxidative stress. However, while this may explain differences between pigmented and non-pigmented strains, it cannot account for the differences among non-pigmented rats with respect to

susceptibility to 2VO.

Electrophysiological analysis of the chronology of events associated with loss of reflex is also warranted. Recording of the photopic electroretinogram (ERG) has shown abnormalities of signal generation and transduction in response to a light stimulus immediately following 2VO (Block et al., 1992). By 7 days of 2VO, complete absence of the ERG b-wave was observed (Barnett and Osborne, 1995). It is possible however that these observations were from eyes which did not lose the pupillary reflex, as this parameter was not assessed in past studies. Assessment of the ERG profile of eyes that show a loss of reflex versus those that do not, conducted at very early time points when loss of reflex occurs, could be insightful.

The mechanisms of neural death, e.g. excitotoxicity, apoptosis *etc.*, also require determination. Considering that the current study suggests that the optic nerve is the site of initial degeneration and breakdown of the pupillary reflex pathway, future studies should investigate mechanisms of white matter degeneration at this site. Ischemia may result in rapid Ca^{2+} mediated toxicity to the myelin sheath which facilitates signal transduction (Stys, 1998). Rarefaction of white matter has been shown to occur as early 7 days following 2VO (Wakita et al., 1995). It would be informative to investigate potential differences in white matter morphology of the optic nerve in 2VO-flex versus 2VO-noflex groups, particularly at early time points when reflex is lost. An *in vitro* electrophysiological investigation of the membrane properties of optic nerve samples from 2VO-flex and 2VO-noflex eyes seems warranted.

It is important to determine if loss of pupillary reflex occurs only in this subset of

Sprague-Dawley rats or if such pathology occurs in other strains of rats at some yet undetected frequency. As well, it is of the utmost importance that pupillary reflex be diligently monitored in future studies employing models of ischemia, since the behavioural consequences are usually assessed by vision-dependent tasks. Past studies, including very recent publications reporting behavioural results from visually guided tasks, have failed to assess the integrity of vision. At very least, some form of visual assessment is required to confirm the validity of the findings when visually guided tasks are employed.

Perhaps a better strategy would be to design tasks which assess the behavioural parameters of interest without relying on visual ability. Studies have shown that rats are able to expertly navigate mazes and perform complex tasks through use of the other sensory modalities, relying on tactile cues (Davidson et al., 2000; Tomie and Whishaw, 1990), olfactory cues (Means et al., 1992; Whishaw and Tomie, 1991), and auditory cues (Sutherland and Dyck, 1984).

For future studies of chronic brain ischemia, a strategy for eliminating the potential for visual system pathology would be to use a model that does not concomitantly induce retinal ischemia. Ligation of the internal carotid artery above the origin of the pterygopalatine artery, as suggested by Ohta et al. (1997), may be one practical solution to this potential problem. However, this is a much more difficult surgical procedure than 2VO.

Clinical Relevance

The 2VO technique can be used to model human neurodegenerative disorders

associated with chronic low-grade ischemia such as carotid artery disease. Carotid artery disease is characterised by partial or complete obstruction of one or both carotid arteries in humans (Dugan and Green, 1991). Visual dysfunction as a result of related retinal ischemia is one of the major and often irreversible symptoms of this disorder. Amaurosis fugax, the most common of such visual disorders, is characterised by transient blindness resulting from bouts of metabolic insufficiency in the retina (Fisher, 1989; Ruben et al., 1990). This is caused by a lack of ocular blood supply, often occurring in combination with exposure to bright light, which increases the metabolic demands of the retina (Dugan and Green, 1991). It is significant to note that the proportion of human patients presenting with visual disturbances ($\sim 2/3$) (Kahn et al., 1986) is similar to the proportion of 2VO rats which suffer from loss of the pupillary reflex accompanied by visual system pathology ($\sim 2/3$). Furthermore, pupillary reflex abnormalities have been noted in cases of carotid artery disease as well (Dugan and Green, 1991; Kahn et al., 1986; Bogousslavsky et al., 1985).

The 2VO model, with the strain of rat used in this study, closely parallels the pathology of human carotid artery disease across a range of symptoms and it provides a useful model for the investigation of the mechanisms of cerebral and retinal degeneration associated with this disorder. It may suggest important steps that could be taken to minimize retinal damage such as reduction of exposure to light. As well, it should be useful in the development and testing of potential therapeutic agents for optic nerve and retinal degeneration, such as antioxidants, which could involve different mechanisms. Therapeutic strategies could first be tested in the 2VO model since as shown here,

ischemic damage to the optic nerve and phototoxic death of the receptors can apparently be dissociated. In this regard, it could be potentially useful to breed a colony of rats with a high or complete incidence of retinal vulnerability to 2VO.

The 2VO model, using the strain of rat in this study, may be a useful alternative to more complicated techniques used for elevated intraocular pressure (IOP) models. The 2VO-noflex eyes suffer substantial neural pathology relatively homogeneously between animals which is likely comparable to that induced in IOP models. The 2VO technique is very simple to execute and has a very low mortality rate (1/74, or ~1.4% in the current study). Disadvantages of IOP models range from difficult surgical techniques requiring ligation of tiny retinal vessels, to invasive intraocular injection requiring sustained anaesthesia for the duration of an experiment. Therefore, the potential value of 2VO as a model for disorders such as glaucoma is worthy of investigation.

Conclusions

2VO surgery caused loss of pupillary reflex as well as retinal and optic nerve degeneration in approximately 58% of a strain of Sprague-Dawley rats. For these eyes, ischemic damage to the optic nerve initiated a cascade of neuropathological events throughout the retina in the following order: (1) Loss of the pupillary reflex to light resulted seemingly from failure of the optic nerve to transduce signals from the eye following ischemic damage. (2) Selective death of RGCs, most likely resulting from retrograde degeneration, virtually eliminated the population of RGCs in the RGCL. (3) Subsequent thinning of the IPL occurred likely as a result of degeneration of the RGC

processes within this layer. (4) The OPL became thinner probably as a result of retrograde degeneration as well, and this was possibly exacerbated in the light condition by degeneration of the photoreceptor axons. (5) In the light condition, photoreceptors were destroyed by light toxicity due to a lack of pupillary reflex.

At the outset of this experiment, it was hypothesized that ischemia in a subset of rats is sufficient such that the metabolic demands of the photoreceptors are not met, resulting in loss of the pupillary reflex. Furthermore, it was hypothesized that phototoxicity would exacerbate retinal and optic nerve degeneration in eyes that lacked the pupillary reflex. In accordance with these hypotheses, it is now concluded that ischemia indeed resulted in loss of pupillary reflex, and that light toxicity did contribute to retinal degeneration in the absence of pupillary reflex. However, the chronology and site of these events were contrary to what was hypothesized. It appears as though the optic nerve, as opposed to the photoreceptors was most susceptible to ischemic injury and was the initial site of failure of the pupillary reflex circuit. Contrarily, the photoreceptors appeared to be relatively resistant to ischemic injury and they slowly succumbed to light toxicity.

REFERENCES

- Barnett N, and Osborne N. Prolonged bilateral carotid artery occlusion induces electrophysiological and immunohistological changes to the rat retina without causing histological damage. *Experimental Eye Research* 1995 61: 83-90.
- Barnstable CJ, and Dräger UC. Thy-1 antigen: A ganglion cell specific marker in rodent retina. *Neuroscience* 1984 11(4): 847-855.
- Bazan NG, Gordon WC, Marcheselli VL, Lukiw WJ, Duhault J, Koenig-Berard E, Linn DM, DeCoster MA, and Mukherjee PK. Experimental models and their use in studies of diabetic retinal microangiopathy. *Thérapie* 1997 52: 447-451.
- Benke T, Neussl D, and Aichner F. Neuropsychological deficits in asymptomatic carotid artery stenosis. *Acta Neurol Scand* 1991 83: 378-381.
- Bennett SAL, Pappas BA, Stevens WD, Davidson CM, Fortin T, and Chen J. Cleavage of amyloid precursor protein elicited by chronic cerebral hypoperfusion. *Neurobiology of Aging* 2000 21: 207-214.
- Bennett SAL, Tenniswood M, Chen J-H, Davidson CM, Keyes MT, Fortin T, and Pappas BA. Chronic cerebral hypoperfusion elicits neuronal apoptosis and behavioral impairment. *Neuropharmacology and Neurotoxicology* 1998 9: 161-166.
- Bennion RS, Owens ML, and Wison SE. The effect of unilateral carotid endarterectomy on neuropsychological test performance in 53 patients. *Journal of Cardiovascular Surgery* 1985 26: 21-26.
- Benson DF, Kuhl DE, Hawkins RA, Phelps ME, Cummings JL, and Tsau SY. The fluorodeoxyglucose ¹⁸F scan in Alzheimer's disease and multi-infarct dementia. *Archives of Neurology* 1983 40: 711-714.
- Blanks JC, Hinton DR, Sadun AA, and Miller CA. Retinal ganglion cell degeneration in Alzheimer's disease. *Brain Research* 1989 501: 364-372.
- Blanks JC, Torigoe Y, Hinton DR, and Blanks RHI. Retinal pathology in Alzheimer's disease. I. Ganglion cell loss in foveal/parafoveal retina. *Neurobiology of aging* 1996 17: 377-384.
- Blanks JC, Schmidt SY, Torigoe Y, Porrello KV, Hinton DR, and Blanks RHI. Retinal pathology in Alzheimer's disease. II. Ganglion cell loss in foveal/parafoveal retina. *Neurobiology of aging* 1996 17: 385-395.

Blesa R, Mohr E, Miletich RS, Hildebrand K, Sampson M, and Chase TN. Cerebral metabolic changes in Alzheimer's disease: Neurobehavioral patterns. *Dementia* 1996 7: 239-245.

Block F, Schwarz M, and Sontag, K-H. Retinal ischemia induced by occlusion of both common carotid arteries in rats as demonstrated by electroretinography. *Neuroscience Letters* 1992 144: 124-126.

Bogousslavsky J, and Regli F. Cerebro-retinal ischemia after bilateral occlusion of internal carotid artery. *Neuroradiology* 1985 27: 238-247.

Casey JE, Ferguson GG, Kimura D, and Hachinski VC. Neuropsychological improvement versus practice effect following unilateral carotid endarterectomy in patients without stroke. *Journal of Clinical and Experimental Neuropsychology* 1989 11: 461-470.

Chan KMC, Young MJ, and Lund RD. Interactive events subserving the pupillary light reflex in pigmented and albino rats. *European Journal of Neuroscience* 1995 7: 2053-2063.

Crawford JG. Alzheimer's disease risk factors as related to cerebral blood flow: Additional evidence. *Medical Hypotheses* 1998 50: 25-36.

Davidson CM, Pappas BA, Stevens WD, Fortin T, and Bennett SAL. Chronic cerebral hypoperfusion: Loss of pupillary reflex, visual impairment and retinal neurodegeneration. *Brain Research* 2000 859(1): 96-103.

De Jong GI, Farkas E, Stienstra CM, Plass JRM, Keijser JN, de la Torre JC, and Luiten PGM. Cerebral hypoperfusion yields capillary damage in the hippocampal CA1 area that correlates with spatial memory impairment. *Neuroscience* 1999 91: 203-210.

de la Torre JC, Fortin T, Park GAS, Butler KS, Kozlowski P, Pappas BA, de Socarraz H, Saunders JK, and Richard MT. Chronic cerebrovascular insufficiency induces dementia-like deficits in aged rats. *Brain Research* 1992 582: 186-195.

de la Torre JC, and Mussivand T. Can disturbed brain microcirculation cause Alzheimer's disease? *Neurological Research* 1993 15: 146-153.

de la Torre JC, Cada A, Nelson N, Davis G, Sutherland RJ, and Gonzalez-Lima F. Reduced cytochrome oxidase and memory dysfunction after chronic brain ischemia in aged rats. *Neuroscience Letters* 1997 223: 165-168.

de la Torre JC. Critical threshold cerebral hypoperfusion causes Alzheimer's disease? *Acta Neuropathol* 1999 98: 1-8.

Dugan JD Jr., and Green WR. Ophthalmologic manifestations of carotid occlusive disease. *Eye* 1991 5: 226-238.

Eklöf B, and Siesjö BK. The effect of bilateral carotid artery ligation upon the blood flow and the energy state of the rat brain. *Acta Physiologica Scandinavica* 1972 86: 155-165.

Eklöf B, and Siesjö BK. Cerebral blood flow in ischemia caused by carotid artery ligation in the rat. *Acta Physiologica Scandinavica* 1973 87: 69-77.

Fisher CM. 'Transient monocular blindness' versus 'amaurosis fugax'. *Neurology* 1989 39: 1622-1624.

Fujishima M, Ishitsuka T, Nakatomi Y, Tamaki K, and Omae T. Changes in local cerebral blood flow following bilateral carotid occlusion in spontaneously hypertensive and normotensive rats. *Stroke* 1981 12: 874-876.

Funk RHW. Blood supply of the retina. *Ophthalmic Research* 1997 29: 320-325.

Gallyas F, Hsu M, and Buzsaki G. Delayed degeneration of the optic tract and neurons in the superior colliculus after forebrain ischemia. *Neuroscience Letters* 1992 144: 177-179.

Gallyas F, Güldner FH, Zoltay G, and Wolff JR. Golgi-like demonstration of "dark" neurons with an argyrophil iii method for experimental neuropathology. *Acta Neuropathologica* 1990 79: 620-628.

Green EC. Anatomy of the rat. Hafner Publishing Co., Inc., New York 1935.

Gundersen HJG, and Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* 1987 147: 229-263.

Haynes CD, Gideon DA, King GD, and Dempsey RL. The improvement of cognition and personality after carotid endarterectomy. *Surgery* 1976 80: 699-704.

Iragui VJ, Kalmijn J, Plummer DJ, Sample PA, Trick GL, and Freeman WR. Pattern electroretinograms and visual evoked potentials in HIV infection: Evidence of asymptomatic retinal and postretinal impairment in the absence of infectious retinopathy. *Neurology* 1996 47: 1452-1456.

Islam N, Aftabuddin M, Moriwaki A, and Hori Y. Detection of DNA damage induced by apoptosis in the rat brain following incomplete ischemia. *Neuroscience Letters* 1995 188: 159-162.

Johnson EC, Deppmeier LMH, wentzien SKF, Hsu I, and Morrison JC. Chronology of optic nerve head and retinal responses to elevated intraocular pressure. *Investigative Ophthalmology and Visual Science* 2000 41(2): 431-442.

Kahn M, Green WR, Knox DL, and Miller NR. Ocular features of carotid occlusive disease. *Retina* 1986 6(4): 239-252.

Kalaria RN. Cerebral vessels in ageing and Alzheimer's disease. *Pharmacol Ther* 1996 72(3): 193-214.

Kalaria RN, and Hedera P. Differential degeneration of the cerebral microvasculature in Alzheimer's disease. *NeuroReport* 1995 6: 477-480.

Kandel ER, Schwartz JH, and Jessell TM. Principles of Neural Science. Appleton & Lange, New Jersey 1991.

Kelly MP, Garron DC, and Javid H. Carotid disease, carotid endarterectomy, and behaviour. *Archives of Neurology* 1980 37: 743-748.

Kondo Y, Ogawa N, Asanuma M, Matsuura K, Nishibayashi S, and Iwata E. Preventative effects of bifemelane hydrochloride on decreased levels of muscarinic acetylcholine receptor and its mRNA in a rat model of chronic cerebral hypoperfusion. *Neuroscience Research* 1996 24: 409-414.

Legg CR. Effects of subcortical lesions on the pupillary reflex in the rat. *Neuropsychologia* 1975 13: 373-376.

Means LW, Alexander SR, and O'Neal MF. Those cheating rats: Male and female rats use odor trails in a water-escape "working memory" task. *Behavioral and Neural Biology* 1992 58: 144-151.

Morris RGM, Garrud P, Rawlins JN, and O'Keef J. Place navigation impaired in rats with hippocampal lesions. *Nature* 1982 297: 681-683.

Ni J-W, Matsumoto K, and Watanabe H. Tetramethylpyrazine improves spatial cognitive impairment induced by permanent occlusion of bilateral common carotid arteries or scopolamine in rats. *Japanese Journal of Pharmacology* 1995a 67: 137-141.

Ni J-W, Matsumoto K, Li H-B, Murakami Y, and Watanabe H. Neuronal damage and decrease of central acetylcholine level following permanent occlusion of bilateral common carotid arteries in rat. *Brain Research* 1995b 673: 290-296.

Ni J-W, Ohta H, Matsumoto K and Watanabe H. Progressive cognitive impairment following chronic cerebral hypoperfusion induced by permanent occlusion of bilateral carotid arteries in rats. *Brain Research* 1994 653: 231-236.

O'Steen KW, Spencer RL, Bare DJ, and McEwen BS. Analysis of severe photoreceptor loss and Morris water-maze performance in aged rats. *Behavioural Brain Research* 1995 68: 151-158.

Ohta H, Nishikawa H, Kimura H, Anayama H, and Miyamoto M. Chronic cerebral hypoperfusion by permanent internal carotid ligation produces learning impairment without brain damage in rats. *Neuroscience* 1997 79: 1039-1050.

Oldendorf WH. Trophic changes in the arteries at the base of the rat brain in response to bilateral common carotid ligation. *Journal of Neuropathology and Experimental Neurology* 1989 48: 534-547.

Olton DS, and Papas BC. Spatial memory and hippocampal function. *Neuropsychology* 1979 17: 669-682.

Osborne NN, Rukhsana S, and Nash M. Photoreceptors are preferentially affected in the rat retina following permanent occlusion of the carotid arteries. *Vision Research* 1999 39: 3995-4002.

Pantoni L, Garcia JH and Gutierrez JA. Cerebral white matter is highly vulnerable to ischemia. *Stroke* 1996 27: 1641-1647.

Panza F, Solfrizzi V, Della T, Nardo GA, Mastroianni F, Lauriero F, D'addabbo A, and Capurso A. Correlation between neuropsychometric tests and semi-quantitative evaluation of the regional cerebral blood flow (rCBF) in a group of outpatients affected by cognitive impairment. *Arch Gerontol Geriatr, suppl* 1996 5: 57-61.

Pappas BA, de la Torre JC, Davidson CM, Keyes MT, Fortin T. Chronic reduction of cerebral blood flow in the adult rat: Late-emerging CA1 cell loss and memory dysfunction. *Brain Research* 1996 708: 50-58.

Parker JC, Smarr KL, Granberg BW, Nichols WK, and Hewett JE. Neuropsychological parameters of carotid endarterectomy: A two year prospective analysis. *Journal of Counselling and Clinical Neuropsychology* 1986 54: 676-681.

Pietrini P, Furey ML, Graff-Radford N, Freo U, Alexander GE, Grady CL, Dani, Mentis MJ, and Schapiro MB. Preferential metabolic involvement of visual cortical areas in a subtype of Alzheimer's disease: Clinical implications. *The American Journal of Psychiatry* 1996 153: 1261-1268.

- Perry VH, Morris RJ, and Raisman G. Is Thy-1 expressed only by ganglion cells and their axons in the retina and optic nerve? *Journal of Neurocytology* 1984 13: 809-824.
- Pourcho RG. Neurotransmitters in the retina. *Current Eye Research* 1996 15: 797-803.
- Ruben JR, McIntyre M, Lukens MC, Plecha EJ, and Bernhard VM. Carotid endarterectomy for chronic retinal ischemia. *Surgery, Gynecology & Obstetrics* 1990 171:497-501.
- Rukhsana S and Osborne NN. Retinas from albino rats are more susceptible to ischaemic damage than age-matched pigmented animals. *Brain Research* 2000 862: 36-42.
- Scinto LFM, Wu CK, Firla KM, Daffner KR, Saroff D, and Geula C. Focal pathology in the Edinger-Westphal nucleus explains pupillary hypersensitivity in Alzheimer's disease. *Acta Neuropathology* 1999 97: 557-564.
- Scremin OU. Cerebral vascular system. In Paxinos, G. (Ed.), *The Rat Nervous System*, 2nd ed. Academic Press, Inc., San Diego 1995 3-35.
- Sefton AJ, and Dreher B. Visual system. In Paxinos, G. (Ed.), *The Rat Nervous System*, 2nd ed. Academic Press, Inc., San Diego 1995 833-898.
- Spencer RL, O'Steen WK, and McEwen BS. Water maze performance of aged Sprague-Dawley rats in relation to retinal morphological measures. *Behavioural Brain Research* 1995 68: 139-150.
- Stys PK. Anoxic and ischemic injury of myelinated axons in CNS white matter: From mechanistic concepts to therapeutics. *Journal of Cerebral Blood Flow and Metabolism* 1998 18: 2-25.
- Stys PL, Waxman SG and Ransom BR. Ionic mechanisms of anoxic injury in mammalian white matter: Role of Na⁺ channels and Na⁺-Ca²⁺ exchanger. *Journal of Neuroscience* 1992 12: 430-439.
- Su JH, Anderson AJ, Cummings BJ, and Cotman CW. Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Clinical Neuroscience and Neuropathology* 1994 5: 2529-2533.
- Sutherland RH and Dyck RH. Place navigation by rats in a swimming pool. *Canadian Journal of Psychology* 1984 38(2) 322-347.
- Taghavy A, and Hamer G. Symptomatic and asymptomatic high-grade unilateral carotid stenosis: scalp topography of event-related potentials (p300) and psychometric testing. *Electroencephalography and Clinical Neurophysiology* 1995 94: 163-174.

Tanaka K, Ogawa N, Asanuma M, Kondo Y, and Nomura M. Relationship between cholinergic dysfunction and discrimination learning disabilities in Wistar rats following chronic cerebral hypoperfusion. *Brain Research* 1996 729: 55-65.

Thome J, Frolich L, Scheubeck M, Hibnauer G, Becker T, Foley P, Wiesbeck GA, Vince GH, Maurer K, and Rosler M. Left hemispheric reductions of regional cerebral blood flow (rCBF) correlate with measures of dementia severity in patients with Alzheimer's disease: A SPECT study. *European Psychiatry* 1996 11: 314-318.

Tomie J and Whishaw IQ. New paradigms for tactile discrimination studies with the rat: Methods for simple, conditional, and configural discriminations. *Physiology and Behavior* 1990 48:225-231.

Tomie J and Whishaw IQ. New paradigms for tactile discrimination studies with the rat: Methods for simple, conditional, and configural discriminations. *Physiology and Behavior* 1990 48:225-231. Tsuchiya M, Sako K, Yura S, and Yonemasu Y. Cerebral blood flow and histopathological changes following permanent bilateral carotid artery ligation in Wistar rats. *Experimental Brain Research* 1992 89: 87-92.

Wakita H, Tomimoto H, Akiguchi I, and Kimura J. Protective effect of cyclosporin A on white matter changes in the rat brain after chronic cerebral hypoperfusion *Stroke* 1995 26(8): 1415-1422.

Wakita H, Tomimoto H, Akiguchi I, and Kimura J. Dose-dependent, protective effect of FK506 against white matter changes in the rat brain after chronic cerebral ischemia. *Brain Research* 1998 792: 105-113.

Wakita H, Tomimoto H, Akiguchi I, Lin J-X, Miyamoto K, and Oka N. A cyclooxygenase-2 inhibitor attenuates white matter damage in chronic cerebral ischemia. *NeuroReport* 1999 19: 1461-1465.

Wässle H, and Boycott BB. Functional architecture of the mammalian retina. *Physiological Reviews* 1991 95: 1-7.

West MJ. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *Trends in Neuroscience* 1999 22: 51-61.

Whishaw IQ and Tomie J. Acquisition and retention by hippocampal rats of simple, conditional, and configural tasks using tactile and olfactory cues: Implications for hippocampal function. *Behavioral Neuroscience* 1991 105(6): 787-797.

Yamamuro Y, Iwano H, Sensui N, Hori K, and Nomura M. Acetylcholine in the hippocampus during the discrimination learning performance in the rat model of chronic cerebral ischemia. *NeuroReport* 1996 7: 1837-1840.

Yung C-W, Harris A, Massicotte S, Chioran G, Krombach G, Danis R, and Wolf S.
Retinal blood flow indices in patients infected with human immunodeficiency virus. *British Journal of Ophthalmology* 1996 80(8): 723-727.

APPENDIX A.1

ANOVA Summary Table for Post-Surgical Reflex Condition

Source	df	F	p
Day	2	.087	.917
Light	1	1.470	.231
Day $\times$ Light	1	.974	.385
Error	47		

APPENDIX A.2

ANOVA Summary Table for Neuron Population Estimate in the RGCL

Source	df	F	p
Group	2	18.18	.000
Day	2	4.70	.014
Light	1	.148	.702
Group $\times$ Day	4	2.214	.083
Group $\times$ Light	2	9.36	.400
Day $\times$ Light	2	11.59	.323
Group $\times$ Day $\times$ Light	4	17.48	.157
Error	44		

APPENDIX A.3

ANOVA Summary Table for Thy-1 IR in the RGCL

Source	df	F	p
Group	2	18.18	.000
Light	1	4.70	.019
Group $\times$ Light	2	.148	.250
Error	16		

APPENDIX A.4

ANOVA Summary Table for Outer Nuclear Layer Thickness

Source	df	F	p
Group	2	1.35	.281
Day	2	16.98	.000
Light	1	.010	.920
Group × Day	4	3.949	.008
Group × Light	2	1.674	.198
Day × Light	2	8.502	.001
Group × Day × Light	4	3.167	.022
Error	47		

APPENDIX A.5

ANOVA Summary Table for Outer Plexiform Layer Thickness

Source	df	F	p
Group	2	12.073	.000
Day	2	26.291	.000
Light	1	.215	.645
Group × Day	4	4.4841	.002
Group × Light	2	1.282	.287
Day × Light	2	.837	.439
Group × Day × Light	4	1.279	.292
Error	47		

APPENDIX A.6

ANOVA Summary Table for Inner Nuclear Layer Thickness

Source	df	F	p
Group	2	2.202	.122
Day	2	4.537	.016
Light	1	.188	.667
Group × Day	4	.697	.598
Group × Light	2	.143	.867
Day × Light	2	3.390	.042
Group × Day × Light	4	1.506	.016
Error	47		

APPENDIX A.7

ANOVA Summary Table for Inner Plexiform Layer Thickness

Source	df	F	p
Group	2	8.589	.001
Day	2	5.671	.006
Light	1	.929	.340
Group × Day	4	2.571	.050
Group × Light	2	.404	.670
Day × Light	2	2.038	.142
Group × Day × Light	4	1.323	.275
Error	47		

APPENDIX A.8

ANOVA Summary Table for Gallyas Silver Staining in the Optic Nerve

Source	df	F	p
Group	2	115.448	.000
Day	2	1.869	.166
Light	1	.080	.778
Group × Day	4	1.132	.354
Group × Light	2	.005	.995
Day × Light	2	1.668	.200
Group × Day × Light	4	1.042	.396
Error	45		

APPENDIX A.9

ANOVA Summary Table for GFAP IR in the Optic Nerve

Source	df	F	p
Group	2	.151	.860
Day	2	.398	.674
Light	1	.308	.581
Group × Day	4	2.471	.058
Group × Light	2	1.484	.238
Day × Light	2	.138	.871
Group × Day × Light	4	.945	.447
Error	45		

APPENDIX B

Summary Table for Scores on the Dependent Measures for Subgroups
of Sham Eyes Which Comprised the CON Group
(n=2 for all cells except where *italics* indicate n=1)

Dependent Measure Scores for Subgroups of Sham Eyes in the CON Group						
	3-DAY		15-DAY		90-DAY	
Dependent Measure	Light	Dark	Light	Dark	Light	Dark
Cell Population in RGCL ($\times 10^3$)	319.1	304.8	226.7	301.4	326.5	340.8
%Area Thy-1 in Retina					1.48	2.0
Thickness of ONL (μm)	29.6	26.6	23.3	23.4	21.02	24.6
Thickness of OPL (μm)	5.8	6.1	5.5	6.0	4.56	5.6
Thickness of INL (μm)	15.0	14.8	13.0	13.9	12.0	12.8
Thickness of IPL (μm)	24.8	25.7	24.8	24.7	24.74	25.9
% Area Gallyas in Optic Nerve	0	0	0	0	0	0
% Area GFAP in Optic Nerve	46.5	42.5	36.5	53.5	37.4	44.5