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**Visual System Pathology May Underlie Chronic Ischemia-Induced
Deficits in Spatial Memory Tasks in the Rat**

by

Christopher Malcolm Davidson, B.A., B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment 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
August 28, 1997
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The undersigned recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis

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Deficits in Spatial Maze Tasks in the Rat"**

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Thesis Supervisor, Bruce A. Pappas, Ph.D.

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Carleton University
September 12, 1997

ABSTRACT

At ~12 months of age, rats pre-trained on the radial maze underwent permanent bilateral common carotid artery occlusion or sham surgery. Forty-four percent (7/16) of occluded animals lost their pupillary reflex (2VO-noflx). Post-surgically, animals were tested weekly on the radial maze. Unlike animals with the reflex (2VO-flx) who never differed from controls, these animals consistently performed more poorly after 6 weeks. After ~30 weeks, 2VO-noflx animals performed more poorly than 2VO-flx and control animals on explicitly visual tasks. After ~41 weeks 2VO-noflx animals did not have greater hippocampal pathology than 2VO-flx animals and controls (cell number and GFAP), but did have more optic tract (GFAP and Gallyas silver stain) and retinal (photoreceptor loss and cell loss in the ganglion cell layer) pathology. It was concluded that chronic ischemia resulted in visual system pathology which may limit the interpretation of spatial memory tasks.

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INTRODUCTION

The mechanisms of neural damage following stroke and myocardial infarction have been explored with experimental ischemia models typically involving the temporary occlusion of one or more of the ascending arteries servicing the brain. Comparably less research has been conducted into the effects of chronically reduced cerebral blood flow despite the parallels with carotid artery disease and various forms of dementia, including Alzheimer's. This project aimed to develop a better understanding of the histological and behavioural effects of chronically reduced cerebral blood flow (CBF). This was accomplished by permanently occluding the common carotid arteries in rats, examining the temporal development of deficits in a spatial maze task and behavioural testing of their visual ability. The animal's brains and retinas were then examined histologically.

Cerebral Blood Flow and Carotid Occlusion

The majority of the brain's arterial blood supply is provided by the internal carotid and vertebral arteries (for review see Scremin, 1995) (Figure 1). The vertebral arteries join and form the basilar artery which runs along the surface of the brain stem and cerebellum. The basilar artery bifurcates and forms the left and right superior cerebellar arteries from which the left and right posterior cerebral arteries stem. The longitudinal hippocampal artery with its short transverse branches originates at the posterior cerebral artery. Vascular supply to the dorsal hippocampus is also

provided by the transverse collicular arteries which also stem from the posterior cerebral artery.

The common carotid artery, bifurcates into the external and internal carotids. The former provides oxygenated blood to the facial and angular arteries. The latter, after giving rise to the pterygopalatine artery, ascends to the base of the brain. Both the middle cerebral, and anterior cerebral arteries originate from the internal carotid.

The posterior communicating artery connects the internal carotid with the posterior cerebral artery completing the anastomoses between arterial blood from the vertebral and internal carotid arteries. This is an important part of the circle of Willis, a structure which connects arterial blood from the different ascending paths.

Permanent bilateral occlusion of the common carotid arteries (2-vessel occlusion; 2VO) results in a reduction in CBF. As measured by metabolic parameters (the difference between arterial and venous blood gas levels) and the ^{14}C -antipyrine method, 2VO reduces CBF to approximately 50 percent of control level shortly after carotid occlusion (Eklöf and Siesjö, 1972; 1973). Similar findings were found using the hydrogen clearance method where 2VO effected a reduction to 40 percent in cortex and thalamus (Fujishima, et al., 1981). In agreement with these results, ^{14}C -antipyrine autoradiographic analysis showed CBF to be 51, 42, 30, and 37 percent of controls in Ammon's horn, caudate, parietal and frontal cortex respectively, 2.5 hours after occlusion (Tsuchiya, et al., 1993). Generally, 2VO results in CBF reductions to approximately 50 percent

Figure 1A: Sagittal representation of the arteries servicing the eyes and brain originating at the aorta. Blackened vessels are parent vessels and main branches of the vertebral, and external carotid artery. * represents approximate point of carotid ligature. Figure adapted from Scremin (1995).

Figure 1B: Bottom projection of the arteries servicing the eyes and brain, highlighting the circle of Willis at the base of the brain. Blackened vessels represent parent vessels and main branches of the circle of Willis. 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**; mcer, middle cerebral artery; olfa, olfactory artery; **pcer, posterior cerebral artery**; **pcom, posterior communicating artery**; ptg, pterygiod artery; **ptgpal, pterygopalatine artery**; sbcl, subclavian artery; **scha, superior cerebellar artery**; vbr, vibrissal arteries; **vert, vertebral artery**; vsp, ventral spinal artery.

of controls shortly after occlusion.

Recently, Ohta et al. (1997) using a novel microsphere infusion technique, found significant reductions of CBF in the anterior (~55% of control) and posterior cortex (~50%), hippocampus (~65%) and striatum (~65%) 2 days after 2VO. Reductions were similar after 10 days of 2VO though slightly lesser in magnitude in the anterior (~60% of control) and posterior (~65%) cortex as well as in the striatum (~70%). Tsuchiya et al. (1993) found that at 1 week following 2VO CBF is generally higher than that after 2.5 hours suggesting the emergence of a process resulting in the attenuation of CBF reduction. At 1 week, 2VO reduced blood flow in Ammon's horn (78% of control), caudate (64%), parietal (60%), and frontal cortex (74%) respectively.

CBF reductions after 2VO continue to diminish with time. Using the hydrogen clearance method after 6 weeks of 2VO CBF in the frontal cortex was 49 percent of controls (Tanaka et al., 1996). This is a smaller reduction than that found with the same method shortly after occlusion (40% of control; Fujishima et al., 1981). Analysis of CBF with the microsphere method after 3 months of ligation found reductions to approximately 80, 80, 83, 76 percent of controls in the anterior and posterior cortices, hippocampus and striatum respectively (Ohta et al., 1997). These reductions were not statistically different from controls.

This increase in regional CBF may reflect the formation of collateral arteries shunting the occlusion. Oldendorf (1989) found that 2VO resulted in basilar and vertebral artery tortuosity which is maximal at 2-4 weeks post-ligation. This tortuosity was slightly

diminished at 15 weeks when numerous small collaterals were seen in the neck, presumably restoring CBF to some degree.

Carotid Occlusion and Cerebral Pathology

Models of acute global ischemia which effect a near total, but brief loss of CBF result in delayed hippocampal pyramidal cell death in the CA1 sector 1 to 10 days later (Auer et al., 1989; Gionet et al., 1991; Nunn and Hodges, 1994). On the other hand, 2VO results in CA1 cell loss after a longer latency. Pappas et al. (1996b) saw a reduction in the number of CA1 pyramidal cells after 190 days of occlusion, but not after 14 days. Similarly, increases in glial fibrillary acidic protein (GFAP), a marker of neural damage (O'Callaghan, 1988), were seen only at 190 days. Others have noted that 2VO results in CA1 pyramidal cell loss after 3-4 months (Ohta et al., 1997; Ni et al., 1994; Ni et al., 1995a), but not after 1 month (de la Torre et al., 1997; Ni et al., 1994; Ni et al., 1995a). Thus, CA1 cell loss emerges between 1 and 3-4 months. Pyramidal cell loss has not been assessed following longer periods of occlusion.

Damage to other brain areas following 2VO has also been noted. Ni et al. (1995a) saw cortical and striatal infarction in 29 and 43 percent of occluded animals respectively after 1 month of occlusion, and 14 and 43 percent respectively after 4 months. Rarefaction in the corpus callosum has been noted in 14 and 71 percent of animals after 1 and 4 months of occlusion (Ni et al., 1995a). Another study reports that carotid occlusion of 1 to 5 days duration resulted in subcortical white matter lesions (Ogata, 1976). There have also been

reports of white matter changes in the visual system (see below).

2VO has also been shown to result in changes in the central cholinergic system. Choline acetyltransferase is the synthesis enzyme for acetylcholine. Activity of this enzyme after 3 weeks of carotid occlusion was lessened and increased relative to controls in the frontal cortex and thalamus respectively (Tanaka et al., 1996). No differences were noted in the hippocampus or striatum. These early changes were accompanied by an increase and decrease in muscarinic acetylcholine receptors in the frontal cortex and thalamus respectively. This presumably reflects adaptations to maintain functional cholinergic homeostasis. Enzyme activity after 6 and 9 weeks did not differ from controls in frontal cortex, thalamus, hippocampus and striatum, nor did it differ at 10 weeks in the cortex, hippocampus or caudate (Pappas et al., 1996b). Reductions in muscarinic receptors were noted after 6 weeks of occlusion in the frontal cortex, hippocampus and striatum, suggesting late emerging cholinergic deficits (Kondo et al., 1996; Tanaka et al., 1996).

Direct measurement of acetylcholine and choline levels after 1 month of occlusion found reduced acetylcholine levels in only the striatum and not in the cortex, hippocampus or hypothalamus (Ni et al., 1995a). After 4 months, acetylcholine and choline levels were reduced in the striatum, cortex, and hypothalamus whereas only choline levels were reduced in the hippocampus. These findings also suggest 2VO results in late emerging cholinergic system deficits.

In vivo measurement of acetylcholine levels gathered from a microdialysis probe positioned above the CA1 sector found lower

resting levels of acetylcholine in occluded animals after 30 days of occlusion (Yamamuro et al., 1996). This contradicts Ni's finding that ex vivo acetylcholine levels are not reduced after 1 month. Generally, central cholinergic dysfunction appears to emerge sometime after 1 month of occlusion.

Carotid Occlusion and Learning and Memory

Experimental acute forebrain ischemia in the rat has been shown to result in deficits in spatial memory tasks such as the Morris water maze and radial arm maze. These deficits are thought to be associated with CA1 sector pyramidal cell loss (Nunn and Hodges, 1994; Olsen et al., 1994). Permanent bilateral occlusion of the carotids results in a different profile of performance on these tasks. The radial maze can be configured so that both working and reference memory are measured. Entries into arms that are never baited constitute errors of reference memory while entries into baited arms from which the animal has already retrieved the food are considered errors of working memory (Olton and Papas, 1979).

On an 8-arm radial maze set up to measure both working and reference memory, Sprague Dawley rats trained prior to 2VO did not show any working memory deficits at 1, 3, 9, 15, and 24 weeks (Pappas et al., 1996b). However, these animals had greater reference memory errors than controls after 9, 15, and 24 weeks, but not after 1 or 3 weeks. The emergence of these reference memory deficits may parallel the emergence of CA1 cell loss and/or cholinergic system dysfunction.

A similar late-emerging deficit was reported by Ni et al. (1994,

1995b) who used an 8-arm maze configured to only measure working memory errors. Pre-surgically trained animals demonstrated an initial increase in errors following 2VO, but quickly approached sham error rates after 3-7 days occlusion. The interposition of a 3-minute delay between the fourth and fifth arm entries resulted in an elevated error rate from post-surgical day 8 to 21. When the 2VO animals were tested again after 4 months, higher error rates occurred even in the no delay condition. Animals who were not trained prior to surgery failed to learn the task despite repeated testing from post-surgical day 7 to 21. This finding was extended by Ohta et al. (1997) who found that untrained 2VO animals made more errors than controls when examined 3 months after surgery.

In conclusion, 2VO animals untrained on the radial maze exhibit poorer performance when acquisition occurs after occlusion. Pre-surgically trained animals however do not exhibit early deficits, though elevated error rates are apparent at about 9 weeks of 2VO. The precise time when these deficits emerge has not yet been ascertained.

The water maze has also been used to assess spatial memory in the ischemic rat. Typically this task involves the animal locating a submerged platform in a circular pool with the aid of cues distributed around the testing room. Like the radial arm maze, 2VO animals not trained pre-surgically on this task have longer latencies in finding the hidden platform at 2, 4, 10, 16, 25 weeks after occlusion (Pappas et al., 1996b).

Unlike the findings for the radial maze, animals pre-surgically

trained on the task still exhibited longer latencies (de la Torre et al., 1997). Data from our laboratory has shown pre-surgical training to result in latencies which do not differ from controls after 1 week, but do differ after 4 weeks of 2VO (Pappas et al., 1996a).

Retinal Structure and Arterial Supply

2VO has also been shown to result in physiological and pathological changes in the rat retina. Cells in the retina are arranged in well organized layers (for review see Wässl and Boycott, 1991) (Figure 10). Briefly, working from the back of the retina forward (from the outer to inner retina) signal transduction occurs in the outer layer of the retina by the cone and rod photoreceptors. Photoreceptor perikaryon form the outer nuclear layer. Photoreceptors synapse with horizontal cells and bipolar cell processes in the outer plexiform layer.

Bipolar cell bodies make up most of the inner nuclear layer though somas of other cell types are also found there. Horizontal cell bodies are located on the outer margin while Müller cell soma are amongst the bipolar cell perikaryon. Amacrine soma are located on the inner margin of the inner nuclear layer.

Amacrine, bipolar and retinal ganglion cell (RGC) processes comprise the inner plexiform layer. Cone bipolar cells synapse directly onto RGC fibres while rod bipolar cells synapse onto AII amacrine cells which have synaptic contact with off-cone bipolar cells and off-retinal ganglion cells. The AII cell processes also form gap junctions with on-cone bipolar cells. Müller cell processes span all

layers of the retina.

RGCs and a population of displaced amacrine cell soma make up the ganglion cell layer. Müller cells and astrocytes are the only glial cells in the retina. Astrocytes are confined to the nerve fibre layer of RGC axons. These axons form the optic nerve when they leave the orbit.

Neural connections in the retina are predominately glutamatergic though most neurotransmitters found in brain have also been found in retina (for review see Pouchro, 1996 and Lam, 1997). Stimulation of photoreceptors results in less glutamate being released into the synaptic cleft. Cone on-bipolar cells with the L-AP4 receptor are thus depolarized. Cone off-bipolar cells with non-NMDA glutamate receptors are hyperpolarized. Rod bipolars, like on-bipolars, are depolarized when the retina is stimulated with light. Cone on- and off-bipolars then form glutamatergic synapses with on- and off-retinal ganglion cells, respectively. Rod bipolars do not directly synapse with RGCs. Instead, they synapse with glycinergic AII amacrine cells, which form inhibitory synapses with cone off-bipolar cells, and off-retinal ganglion cells. AII cells also form gap junctions with cone on-bipolar cells resulting in the conservation of the sign (excitatory) of transmission. Thus, the rod pathway communicates with RGCs that also receive signals from the cone pathway. RGCs express the calcium-conducting NMDA receptor (Hartveit et al., 1994).

Retinal Blood Flow and Ischemia

In the rat, arterial blood supply from the common carotid artery

is essential for the eye and its contents (Scremin, 1995). The pterygopalatine artery, which feeds the external ophthalmic artery stems from the internal carotid (Figure 1). Like the pterygopalatine, the internal ophthalmic and trigeminal arteries also stem from the internal carotid artery. Branches from the internal and external ophthalmic, and trigeminal form an anastomotic network at the back of the eye. The eye also receives arterial supply from the angular artery which indirectly originates from the external carotid. In the rat it is the external ophthalmic which supplies most of the vascular supply via the retinal artery. The retinal artery bifurcates with a nasal and temporal branch servicing their respective halves of the retina (Janes and Bounds, 1954). The ciliary artery system, which supplies the choroid, also draws from the ophthalmic artery.

Due to parallels with glaucoma, many experimental rat studies have used elevated intraocular pressure to induce retinal ischemia. Forty-five to 90 minutes of elevated intraocular pressure and subsequent reperfusion consistently results in a loss of RGCs after 1 to 2 weeks (Sellés-Navarro et al., 1996; Hughs, 1991; Lam et al., 1997). RGC loss was minimized in rat and rabbit by the application of NMDA-receptor antagonists before and after the ischemic episode, respectively (Lam et al., 1997; Louzada-Junior et al., 1992). Coupled with the finding that for rabbits, raised intraocular pressure results in a 2-3 fold increase in extracellular glutamate and subsequent reperfusion results in a 5-10 fold increase (Louzada-Junior et al., 1992), this suggests strongly that excitotoxicity may play a

role in RGC death. Electron and light microscopy as well as terminal deoxynucleotidyl transferase-mediated nick end labelling suggest that some RGCs expire via apoptosis (Büchi, 1991; Quigley et al., 1995; Garcia-Valenzuela et al., 1995).

As discussed previously, the carotids indirectly provide most of the arterial supply to the eye though their occlusion is not likely to cause a complete cessation due to the presence of the circle of Willis. The degree of blood flow reduction in the retina has not been measured.

After 30-45 minutes of carotid occlusion, the eletroretinogram b-wave amplitude is smaller, and implicit time is longer (Barnett and Osborne, 1995; Block, 1992). The b-wave is thought to reflect the depolarization of on-bipolar cells and the subsequent activation of Müller cells. This finding, coupled with the fact that protein kinase C immunostaining of bipolar cells is unchanged, suggests that the electrophysiological changes were not a function of bipolar cell loss. This observation was extended to rats with carotid occlusions for 1 week, for whom the b-wave was extinguished (Barnett and Osborne, 1995). Interestingly, the a-wave amplitude of the electroretinogram (a function of photoreceptors hyperpolarizing) is accentuated by carotid occlusion. The authors attributed this to the absence of the b-wave depolarization which moderates the appearance of the a-wave hyperpolarization.

Morphologically, carotid occlusion of 1 week resulted in the increased expression of glial fibrillary acidic protein (GFAP) in Müller cell processes (Barnett and Osborne, 1995). GFAP expression

in Müller cells is usually confined to their endfeet in the ganglion cell layer and has been shown to be upregulated, and expressed in all retinal layers in response to injury (Huxlin et al., 1995; Bignami et al., 1979). No evidence of perikaryon loss of any retinal cell type was seen at this time point.

Examination of the rat retinal vascular system 1 week following chronic bilateral carotid occlusion revealed that the rate of arterial and venous filling was slower than that pre-operatively, and that a patchy distribution of the capillary network appears (Slakter et al., 1984). Hence, in addition to reducing blood flow to the retina, 2VO may result in poor vascularization of that structure.

A study of the effect of 2VO on the optic nerve in pigmented Long Evans rats found that occlusion lasting 16 weeks resulted in a reduction in nerve diameter, the loss of myelinated and non-myelinated fibres and the replacement of these fibres with linear astrocytic processes (Takamatsu et al., 1984). Furthermore, macrophages were seen to contain myelin within their soma.

Wakita et al. (1994) demonstrated in albino Wistar rats that shortly after occlusion, 2VO resulted in an increase of microglial expression of major histocompatibility complexes I and II in white matter including the optic nerve and tract, corpus callosum, internal capsule, anterior commissure and transversing bundles of the caudoputamen. By 7 days occlusion, rarefaction, as well as activated glial cells (more GFAP) and lymphocytes were noted in the optic nerve and tract. Similar changes in other white matter were seen at later dates, but tended to be less dramatic. These increases were still

present after 90 days of occlusion. Unpublished research in our laboratory has found increases in optic tract degeneration after 2 weeks and more extensive degeneration after 40 weeks. Chronic ischemia in a gerbil model found white matter rarefaction and gliosis beginning at 8 weeks (Hattori *et al.*, 1992).

Transient ischemia has been shown to result in optic tract and optic nerve pathology. Gallyas *et al.*, (1992) found optic tract degeneration 2-6 months following 15 minutes of bilateral occlusion of the vertebral and carotid arteries. Similar damage was not seen 1-5 days after ischemia. Transynaptic degeneration was noted in the superior colliculus where some axons of the optic tract terminate. Thus, both acute and chronic ischemia have been shown to result in optic nerve and tract rarefaction and gliosis which does not appear until 7 days or even later.

Pupillary Reflex

The pupillary reflex serves to reduce the amount of light reaching the retina by constricting the pupil, effectively limiting light toxicity to the photoreceptors. The circuitry underlying this reflex starts with transduction by photoreceptors, and later transmission via RGCs whose axons project to the olivary pretectal nucleus. The olivary pretectal nucleus axons then project to and synapse with neurons of the pre-ganglionic parasympathetic Edinger-Westphal nucleus whose axons make up part of the oculomotor nerve (cranial nerve III). These axons then innervate the accessory motor nucleus, which in turn innervates the muscles controlling pupillary

constriction. The ventral lateral geniculate nucleus may also play a role in the reflex as it receives retinal projections and communicates directly with the olivary pretectal nucleus. Bilateral lesions of this structure result in mydriasis (Legg, 1975).

Carotid Occlusion and Vision

Recently, there have been reports of visual discrimination deficits in animals with 2VO. On a task where an animal is reinforced with food by pressing a lever when a light is either on or off, the lever presses of 2VO rats were equally distributed between the correct and incorrect light stimulus. Control animals pressed the lever more frequently during the correct light stimulus. This has been shown from 1 to 30 days (Yamamuro et al., 1996) and at 6 weeks post-surgically (Tanaka et al., 1996). The authors of these studies suggested this was due to brain cholinergic deficits, and did not address visual system pathology. An obvious competing explanation was that the visual system was damaged by 2VO. Further behavioral evidence that 2VO results in visual system detriments, perhaps even blindness is the fact that locomotor activity patterns of these animals does not vary with the light cycle (Ohta et al., 1997).

Clinical Relevance of the 2VO Model

Permanent carotid occlusion in rats may provide important insights into the role of reduced CBF in Alzheimer's disease and more directly into the effects of carotid disease. While Alzheimer's disease (AD) is not commonly associated with carotid occlusion, it is

associated with reductions in CBF (Prohovnik, 1988; Rogers et al., 1986; Deutsch and Tweedy, 1987; Benson et al., 1983). Furthermore, it has been found that reductions in cerebral blood flow predict the rate of decline in Alzheimer's patients (Wolfe et al., 1995).

Both 2VO and AD are associated with progressive deficits in memory tasks and share some similar changes in brain matter. For example, central cholinergic system deficits have long been associated with AD (Davies and Maloney, 1976). In fact, modern pharmacological treatments for AD attempt to activate this system. As reviewed above, 2VO also results in later emerging reductions in markers of cholinergic system function.

It has been suggested that in AD, changes in brain capillaries such as amyloid deposition, basement membrane thickening, reduced vessel calibre and elasticity, effectively disrupt CBF lessening the availability of energy substrates. This in turn may cause ischemia-vulnerable neurons to become distressed, and to stimulate glial cell activation, and amyloid production, setting in motion a degenerative cascade which is seen clinically as AD (de la Torre and Mussivand, 1993).

Until recently, AD-associated spatial memory deficits were attributed exclusively to brain pathology, with particular attention to the hippocampus, and cortex. Recently however, it has been found that visual system deficits are common in AD. Electrophysiological studies have found that the b-wave on the pattern-induced electroretinogram is reduced to 50 percent (Katz, 1989). This may reflect optic nerve and retinal ganglion cell dysfunction or damage.

Electroretinography has also shown physiological deficits in 2VO rats (Barnett and Osborne, 1995; Block, 1992). Post-mortem examination of eyes from patients with AD have found RGC loss accompanied by swollen or shrunken somas, vacuolization, and pyknotic nuclei. Notably absent were the AD hallmarks of amyloid plaques and neurofibrillary tangles (Blanks et al., 1989). AD also results in increased GFAP immunoreactivity in retinal Müller cell endfeet, radial processes, and retinal astrocytes. As one would expect, AD then is also associated with optic nerve degeneration (Hinton et al., 1986). Furthermore, this optic nerve pathology is often seen in association with thickened arterial vessels suggesting this damage may be ischemia related.

2VO is a model of carotid disease in humans. In carotid disease atherosclerotic plaques result in stenosis of the common or internal carotid, resulting in a reduction in cerebral blood flow (Schroeder, 1988). Patients with unilateral or bilateral stenosis have been shown to have cognitive deficits relative to matched controls (Taghavy and Hamer, 1995; Kelly et al., 1986). Furthermore, some studies report that restoration of blood flow via carotid endarterectomy results in cognitive improvement (Bennion et al., 1985; Haynes et al., 1976, Parker et al., 1986), though dissenting studies do exist (Casey et al., 1989; Greiffenstein et al., 1988).

The visual system is affected by carotid stenosis, typically resulting in episodes of amaurosis fugax or transient monocular blindness (Breen, 1991; Dugan and Green, 1991). Amaurosis fugax is commonly attributed to emboli, vasospasm or vascular insufficiency.

More complete stenosis or complete occlusion results in chronic ocular ischemia, typified by the bleeding and formation of microaneurisms in retinal arteries. With the passing of time neovascularization occurs resulting in increased intraocular pressure and neovascular glaucoma. Carotid endarterectomy results in the erradication of amaurosis fugax, but does little to attenuate the damage associated with neovascular glaucoma.

Overview

2VO results in reductions in CBF which lessen in magnitude with time. Conversely, spatial memory deficits in the radial maze show that working and reference memory errors emerge sometime between 3 and 9 weeks post-ligation. Others also report a progressive increase in errors on similar tasks. This may relate to the late emergence of cholinergic deficits. Similarly, hippocampal pyramidal cell loss was noted after 3 to 4 months of occlusion, but not after 1 month. Carotid occlusion has also been shown to result in pathology in the optic nerve, tract and retina which may contribute to poorer performance on behavioral tasks requiring vision.

In order to determine precisely when radial maze deficits emerge, a time course study was begun where animals were trained to an established criteria on a radial maze, had their common carotid arteries permanently ligated, then were re-tested continually for 16 weeks. Animals were then given a 7-week break as continuous training may have minimized group differences. They were then re-tested for one week.

During radial maze testing, we became aware of literature describing visual system changes in 2VO animals (Barnett and Osborne, 1995). From that point, their unilateral pupillary reflex was monitored. Following radial maze testing, the visual system was assessed behaviourally with explicitly visual tasks in the water maze. After the completion of visual assessment, animals were perfused and the brains and eyes were assessed histologically. Among the brain histological measures examined were pyramidal cell number and GFAP immunoreactivity in the hippocampus as well as degeneration and GFAP immunoreactivity in the optic tract. Cell layer thickness and ganglion cell layer nuclei reductions as well as GFAP immunoreactivity were examined in retina. 2VO animals were subdivided into those with, and those without a pupillary reflex. Inferential statistical analyses was performed on all measures. These were followed by correlational analyses within each group.

It was predicted that radial maze deficits would emerge between 3 and 9 weeks after ligation, and that hippocampal CA1 cell loss would be apparent after 41 weeks of 2VO. RGC, optic tract, and photoreceptor degeneration was expected in these animals, especially those lacking a pupillary response. It was reasoned that without a pupillary reflex, photoreceptors would be vulnerable to light toxicity. Those animals without a pupillary reflex were also then expected to perform more poorly than controls on the radial maze, as their visual system was expected to be damaged. Similarly, this group of animals was expected to show deficits in explicitly visual tasks in the water maze.

METHODS

Animals

Approximately 10-month old male retired breeder Sprague Dawley rats (510-711g) were obtained from Charles River. Animals were singly housed with free access to water, in a 12-hour reverse light cycle (on 20:00, off 8:00). All procedures were approved by the Animal Care Committee of Carleton University.

Pupillary Constriction

Pupillary constriction was examined by exposing an animal's dark adapted eye to a beam of light from a hand-held otoscope. Since the possibility that the visual system may be affected by 2VO arose while the experiment was already under way, only 12 of 27 animals had their reflex observed from surgery onwards. Those monitored from surgery were examined weekly for 6 weeks and then before any new behavioural task and before perfusion. The pupillary reflex of the remaining 15 animals was first examined 118 ± 14 days after surgery, then before any new behavioural task and before perfusion. Three groups were formed; animals with ligated common carotid arteries without the pupillary reflex (2VO-noflx), with the reflex (2VO-flx) and sham operated animals (CON).

Radial Arm Maze Training

Prior to surgery, animals were trained on a novel 10-arm radial arm maze to an established performance criterion. The maze was constructed of plastic and was elevated 86 cm from the floor. The grey floor had at its centre a metal dish 3 cm in diameter through which a 10% sucrose solution could be introduced through the underside of the maze. Arms were equally spaced around the centre portion, 37 cm from the centre dish, 66 cm long and 12 cm wide. 21 cm down each arm was a clear plastic door that could be opened and closed remotely. At the end of each arm was a food well, deep enough that the animal could not see if reward was present unless in close proximity. The walls of the testing room were adorned with visual cues constructed of bristol board.

Rats food deprived to $85\% \pm 2.5\%$ of their body weight (with a 3g/week increase permitted) were taught that all the doors opened when the sucrose solution was consumed. Initially, animals were enticed to the ends of the arms by placing fractions of the food reward (Honey Nut Cheerios) along those arms that would eventually contain the food reward (1/2 Honey Nut Cheerio) in the food well. 8 of the 10 arms contained food reward. The baited 8 arms varied across rats and the 2 unbaited arms were always beside each other. Once an animal entered an arm (2 paws past door) more sucrose solution was delivered to the centre of the maze. When an animal exited an arm the doors were closed behind it requiring it to visit the centre again and consume more of the sucrose before the doors were re-opened. A time limit of 15 minutes for the animal to find all 8 food rewards was instituted.

Entries into arms that no longer or never contained a food reward were considered errors. Errors were delineated into two categories, (i) baited arm re-entries (BAR), where an animal enters a baited arm which it had previously visited and (ii) unbaited arm entries (UAE), where an animal enters an arm that never contains a food reward.

Animals were trained for 5 consecutive days with a 2 consecutive day break per week. The criterion for surgery was that in a 3-testing day cluster the rat must (1) finish the task on all three days, and (2) on each of 2 of the days commit no BAR errors and a maximum of 1 UAE. On the other day, animals had to complete the task in at least 11 arm entries.

Two-Vessel Occlusion

Rats that reached criterion were anaesthetized with ketamine hydrochloride (100 mg/kg i.m.) and methohexital sodium (40 mg/kg i.p.). To prevent respiratory distress during surgery, animals were also administered .04 mg atropine sulphate (i.m.). During surgery the animals body temperature was maintained at 37°C via a rectal thermistor probe connected to the power supply of a heat lamp.

A ventral mid-line incision was made, and the common carotid arteries were then exposed bilaterally and gently separated from the carotid sheath and vagus nerve. Animals assigned to the 2VO surgery had their carotid arteries doubly ligated with 5-0 silk suture. Animals were allowed to recover for 3-4 days with *ad lib* access to food before testing was resumed.

Radial Arm Maze Testing

Animals were tested as before for 16 weeks. Testing ceased for 7 weeks after which the animals were tested for 1 week.

Vision Assessment

At 208 ± 41 days post-surgery, animals were tested on visual tasks in the Morris water maze. The water maze was constructed of white plastic, had a diameter of 160 cm and was 61 cm deep filled to a depth of 45.5 cm with water ($21 \pm 1^\circ\text{C}$). The first task was a 2-cue task in which two cylinders 12 cm in diameter and 20 cm long were suspended from the ceiling 32 cm above the water, 28.5 cm from the side of the pool, directly across from each other. One cylinder was black, and the other bright yellow, and their positions were interchangeable. Animals were placed in the pool at its side, facing the pool centre, so that they were an equal distance from both cues. A submerged platform, not visible to the rats was always placed under the black cue. Latencies to reach the platform were recorded as well as which cue was swum under first (first annulus crossing). The animals were tested for 2 blocks of 5 days, 12 days apart. Each day consisted of 9 trials, with the black cue's location varied in a pseudo-random order. Daily averages of platform location latencies and daily percent correct first annulus crossings were calculated. Path length (from video recording) and latency to locate the platform on the first trial were used to calculate swim speed.

Animals without pupillary constriction had diminishing latencies. It was thought that since the platform was always located

at one of two places, these animals may have been utilizing non-visual search strategies. To control for this, the animals were then tested for 5 days (8 trials/day) on a 1-cue task with the black cue located at any location in the pool for any given trial. The platform was located in each quadrant of the maze 2 times per day, thus the daily distance necessary to swim was roughly the same. Again platform location latencies were recorded, and daily averages were calculated.

Tissue Preparation

At 288 ± 28 days post-surgery animals were anaesthetized as described before and transcardially perfused with 75 ml heparinized saline then 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. A suture was placed at the top each eye for orientation when sectioning. Brains and eyes were post-fixed in this fixative solution for 24 hours at 4°C then washed with phosphate buffer, dehydrated in graded alcohols, cleared in clearene and then paraffin embedded.

Tissue Sectioning and Staining

Four and 8 μm coronal brain sections containing the dorsal hippocampus and optic tract approximately -2.8, -3.3, -3.8, -4.3mm from bregma were sectioned with a rotary microtome. 4 and 8 μm cross-sections of the central retina of the left eye were also prepared.

To examine optic nerve damage, 8 μm sections -2.8, -3.3, -3.8mm

from bregma were stained with the Gallyas silver stain (Gallyas et al., 1990). Briefly, sections were de-paraffinized, and incubated overnight in 1-propanol containing 3.2% distilled water, 4.8% (v/v) concentrated sulphuric acid at 56°C. The sections were then hydrated, and reacted with 0.5% glacial acetic acid for 5 minutes. Then they were reacted with developer (100 mls 10% sodium carbonate, 90mls distilled water, 0.25 g ammonium nitrate, 0.20g silver nitrate, 10 mls 20% tungstosilic acid, 0.5mls 37% formaldehyde, .25% glacial acetic acid) for 10 minutes. A 30-minute wash in 1% glacial acetic acid, then a 5-minute wash in propanol were followed by a 5-minute exposure to anise oil.

In order to visualize hippocampal pyramidal cells, 4 µm sections -3.3, -3.8, -4.3mm from bregma were stained with Palmgren's silver stain (Page, 1971). Slides were de-paraffinized, then placed in acidifier for 5 minutes (25 mls 37% formaldehyde, 75 mls distilled water, and 0.2 mls 1% nitric acid), followed by three water washes. Sections were then covered with silver mordant (15 g silver nitrate, 10 g potassium nitrate, 100 ml distilled water, and 1 ml 5% glycine) for 50 minutes, then wiped clean and exposed to reducer (10 g pyrogallol, 450 ml distilled water, 550 ml ethanol, and 2 ml nitric acid), for 1 minute at 45 °C. After washing with tap water, sections were placed in a 5% sodium thiosulphite solution for 5 minutes. After another tap water wash, sections were dehydrated, and cleared with ethanol and toluene washes.

Central retinal sections were stained with hematoxylin and eosin. Briefly, following de-paraffinization, sections were incubated

in ammonical ethanol (2.8% ammonium hydroxide in 95% ethanol) for 10 minutes, then washed in cold running water for 2 minutes. A 3-minute incubation in Mayer's hematoxylin (2.5% hematoxylin, 10% ethanol, 4.5% aluminum ammonium sulphate, .02% sodium iodate, .1% citric acid) at 60°C was followed by a 3-minute wash in warm tap water. Sections were differentiated in acid-alcohol, washed in warm water, then incubated for 8 seconds in a 60°C eosin solution (.27% eosin). Slides were then washed with water, dehydrated, cleared and coverslipped.

Finally, 4 μ m sections of brain (-2.8, -3.3, -3.8, -4.3mm from bregma) and central retina were stained immunohistochemically, with a monoclonal antibody to glial fibrillary acidic protein (GFAP). Sections were de-paraffinized and re-hydrated, then reacted with 1:600 mouse anti-GFAP (Sigma) in a blocking buffer, followed by biotinylated goat anti-mouse antibody (1:100) (Amersham), then streptavidin peroxidase (1:100) (Amersham). Slides were then presoaked for 5 minutes in a solution containing .02% 3,3 diaminobenzidine, .6% ammonium nickel sulphate and .76% trizma pre-set crystals (pH 7.4), after which 2.4 μ l of hydrogen peroxide were added to the solution. Slides were allowed to incubate in that solution for 7 minutes after which another 2.4 μ l of hydrogen peroxide was added and slides were incubated for further 7 minutes. Sections were then counterstained with .17% pyronin Y in a .05N acetate buffer, pH 4.8. Finally the slides were then dehydrated, cleared and coverslipped.

Histological Measures

All hippocampal measures were derived from 2 sections at each

of -3.3, -3.8 and -4.3mm from bregma. Optic tract measures were derived from 2 sections at each of -2.8, -3.3, -3.8mm from bregma. Averages across sections and plates were calculated. All microscopic investigations were carried out by an observer blind to group membership on an Olympus BH-2 microscope. The total number and number damaged left and right hippocampal CA1 and CA4 pyramidal cells were counted manually from silver stained sections. Damaged cells had smaller, darker nuclei with an irregular shape. GFAP immunoreactivity (percent area) was measured in the oriens layer of the left and right hippocampus (CA1 and CA4 sectors), and in the left and right optic tract with image analysis software (Imaging Research, St. Catherine's) at 200X magnification. Gallyas staining of the optic nerve was similarly analyzed.

The same software was used to measure the thickness of the different layers of central retina, stained with hematoxylin and eosin. Density of nuclei in the ganglion cell layer was also noted. Retinal GFAP immunoreactivity in the different layers of central retina was assessed along a scale where 0 represented no immunoreactivity and 4 represented extensive immunoreactivity. All retinal measures were conducted on the medial, nasal and temporal aspects of the central retina. Nasal and temporal aspects were operationally defined as being the section of the retina closest to the ciliary body where retinal thickness appears stable.

RESULTS

Surgery and Pupillary Reflex

Of the 27 animals trained to criterion on the radial arm maze, 11 received the sham surgery and formed the control group (CON). The remaining 16 animals had both common carotids ligated.

Since the possibility that the visual system may be affected by 2VO became apparent while the experiment was already under way, only 12 animals had their reflex observed from surgery onwards. 4 of 8 of these occluded animals lost their reflex within 1 week post-ligation. In the 12 animals whose pupillary reflexes was monitored from approximately 16 weeks post-ligation, a similar profile occurred; 3 of 8 animals with 2VO lost the reflex. In total, 7 of 16 2VO rats lost their pupillary reflex. All CON animals retained the reflex.

As the occluded animals monitored from surgery soon lost their reflex following ligation and no animals lost or regained the reflex at later post-surgical time points, those animals first inspected at the end of radial maze testing were grouped together with animals who were shown to lose the reflex shortly after surgery. Further support that pupillary reflex loss only occurs shortly after 2VO has been found in two separate studies ongoing in this laboratory (Bennett et al., in preparation. Pappas et al., in preparation).

Radial Arm Maze

Animals had to reach a performance criterion prior to surgery, and were subsequently tested on the radial maze for 16 weeks, then

again after a 7- week break. Average daily BAR and UAE errors were calculated for each post-surgical week and used for statistical analysis.

Baited Arm Re-Entries

Analysis of variance with group as a between subjects variable (CON, 2VO-flx and 2VO-noflx), and post-surgical week (1-16) as a repeated measure found a significant group effect ($F(2,20) = 6.64$, $p < .01$) (Figure 2). *Post-hoc* analysis with Scheffé's test found that only the 2VO-noflx group had more BARs than the CON ($p < .05$) and that the 2VO-noflx and 2VO-flx groups did not differ from each other. No general difference was noted across post-surgical week, nor was there a significant group by week interaction.

In order to gauge the effect of a 7-week break in testing, a pre-post ANOVA was performed with post-surgical week 16 average BAR errors constituting the 'pre' values. The groups were not uniquely affected by the break, though generally animals made more BAR errors after the break ($F(1,19) = 4.88$, $p < .01$). *Post-hoc* analysis again found the 2VO-noflx group, but not the 2VO-flx group made more BAR errors than the CON group ($p < .05$). The analysis also found that the two 2VO groups did not differ from each other.

A priori ANOVAs at each post-surgical week were conducted, followed by Scheffé's test for those weeks with significant group differences. The 2VO-noflx group committed more BAR errors compared

Figure 2. Radial maze baited arm re-entry errors. Overall, the groups differed ($F(2,20) = 6.64$, $p < .01$), with the 2VO-noflx group making more BAR errors than the CON group ($p < .05$, Scheffé's test). Significant group differences at each week were examined (* and ** represent differences between the 2VO-flx and CON groups at the $p < .05$ and $p < .01$ levels respectively, Scheffé's test; \$ represents differences between the 2VO-noflx and 2VO-flx groups at the $p < .05$ level. Error bars represent S.E.M.

to the CON group at post-surgical weeks 2, 6, 10, 11, 12, 14, 15 and 23 ($p < \text{at least } .05$). The 2VO-noflx animals committed more BAR errors than the 2VO-flx group only on post-surgical weeks 2 and 15 ($p < .05$).

Unbaited Arm Entries

Analysis of variance with group as a between subjects variable and post-surgical week as a repeated measure revealed a significant group effect ($F(2,20) = 6.02$, $p < .01$) (Figure 3). Scheffé's *post-hoc* test revealed that the 2VO-noflx group had significantly more UAEs than the CON or the 2VO-flx groups ($p < .05$). Across the three groups, UAE errors changed over the weeks of testing ($F(15,300) = 3.02$, $p < .001$).

Examination of the effect of the break in testing again revealed a significant group effect ($F(2,19) = 10.50$, $p < .01$). *Post-hoc* analysis found the 2VO-noflx group made more errors than that both the CON group and the 2VO-flx group ($p < .01$ and $p < .05$, respectively). The groups were not affected differently by the break, though a general increase in the number of errors was noted ($F(1,19) = 7.86$, $p < .05$).

A *priori* analysis of group differences followed by Scheffé's test where appropriate at each post-surgical week were performed. The 2VO-noflx group committed more UAEs than the CON group on post-surgical weeks 6 to 16 and 23 ($p < .05$), while the 2VO-noflx group committed more than the 2VO-flx group at post-surgical weeks 3, 7, 9, 11, 13, 16 and 23 ($p < .05$).

Figure 3. Radial maze unbaited arm entry errors. Overall, the groups differed ($F(2,20) = 6.02$, $p < .01$), with the 2VO-noflx group making more UAE errors than the CON group ($p < .01$, Scheffé's test). Significant group differences at each week were examined (* and ** represent differences between the 2VO-flx and CON groups at the $p < .05$ and $p < .01$ levels respectively, Scheffé's test; \$ and \$\$ represent differences between the 2VO-noflx and 2VO-flx groups at the $p < .05$ and $p < .01$ levels respectively. Error bars represent S.E.M.

Water Maze Vision Assessment

2-Cue Task

At 208 ± 41 (range) days after surgery the animal's visual ability was assessed in the water maze. Two, five-day blocks of testing in the 2-cue task were completed 12 days apart, with each testing day consisting of 9 trials. The hidden platform was always located under the black visual cue, and never under the yellow cue whose fixed positions were alternated in a semi-random order. During testing the latency to locate the platform was recorded, as was the daily percentage of correct first annulus crossings. On the first trial of the first day of testing, swim speeds of the animals were compared and the groups were found not to differ ($F(2,21) = 1.26$, n.s.). Speeds for the CON, 2VO-flx, and 2VO-noflx groups were 23.4 ± 1.4 , 26.1 ± 1.5 and 26.7 ± 1.8 cm/s, respectively (mean $\pm$ S.E.M.).

Analysis of variance of the platform location latencies with group as a between subjects factor and day as a within subjects factor, revealed a significant group effect ($F(2,21) = 25.61$, $p < .001$), with the 2VO-noflx group taking more time to find the platform than either the CON or 2VO-flx groups ($p < .05$) (Figure 4). There was also a significant effect of testing day ($F(4,84) = 3.97$, $p < .01$), reflecting longer latencies at the beginning of this test.

As the platform was always in one of two possible locations during this task, it is possible that animals could minimize platform location latencies using non-visual strategies such as swimming in a '7' pattern, where both possible locations of the platform are swum over. In an attempt to see if the animals were distinguishing

Figure 4. 2-Cue vision task. Error bars represent S.E.M. **Top Panel:** Latencies to locate the platform. Data points are averages of 9 trials on a given day. Generally, latencies differed between groups ($F(2,21) = 25.61$, $p < .001$) with the 2VO-noflx group taking longer than both the 2VO-flx and CON groups ($p < .01$, Scheffé's test). Latencies changed across testing days ($F(9,189) = 2240.35$, $p < .001$), and did so differently across groups ($F(18,189) = 1.66$, $p < .05$). **Bottom Panel:** Daily percent correct first annulus crossings. Groups did not differ on this task ($F(2,21) = 1.97$, $p < .17$), though only the 2VO-noflx group failed to perform this task at better than chance level (t-tests).

between the yellow and black cues, the daily percent correct first annulus crossings were examined (Figure 4). These values were analyzed in the same manner as the platform latencies. The groups did not differ on cue discrimination $F(2,21) = 1.97$, $p = .17$, though generally percentages did differ across days $F(9,189) = 1.98$, $p < .05$. The percent correct for all of the 10 days of testing were averaged and were found to be greater than 50 percent for the CON ($60.0 \pm 1.8\%$; $t(9) = 5.54$, $p < .001$) and 2VO-flx ($57.1 \pm 2.9\%$; $t(9) = 2.44$, $p < .05$) groups though not for the 2VO-noflx group ($52.4 \pm 2.4\%$; $t(4) = .98$, $p < .40$) (mean $\pm$ S.E.M.).

1-Cue Task

To reduce the rat's utilization of non-visual search strategies, a 1-cue task was devised where the black cue and platform were randomly positioned in the pool for a given trial. Animals were tested for 5 days, with 8 trials per day. Analysis of the latencies to find the platform on this task with group as a between subjects variable and day as a within subjects variable revealed a significant group effect ($F(2,21) = 21.88$, $p < .001$) with the 2VO-noflx group taking longer to locate the platform than both the 2VO-flx and CON groups ($p < .01$) (Figure 5). The 2VO-flx group did not differ from the control group. There was also a significant day effect ($F(4,84) = 3.97$, $p < .01$), likely reflecting a generalized shortening of latencies. Thus, the 2VO-noflx group took longer to locate the platform on both the 2- and 1-cue tasks, and this was not likely a function of different swim speeds.

Figure 5. 1-Cue vision task platform location latencies. The group latencies differed ($F(2,21) = 21.88$, $p < .001$), with the 2VO-noflx group taking longer than both the 2VO-flx and CON groups ($p < .01$, Scheffé's test). Across groups latencies varied from day to day ($F(4,84) = 3.97$, $p < .01$). Error bars represent S.E.M.

Behavioural Results - Correlational Analysis

Summary statistics for BAR and UAE errors consisted of an average of daily error rates over post-surgical weeks 14, 15, and 16. Similarly, 2-cue task platform location latencies over the last two days on the 2-cue task were averaged, while the final day's average latency was used as a summary statistic for the 1-cue task. BAR and UAE were correlated for both the CON ($r=.69$ $p<.05$) and 2VO-flx ($r=.83$, $p<.01$) groups, though not for the 2VO-noflx group ($r=.60$, $p<.16$) (Figure 6). Significant correlations were also found for the CON and 2VO-flx groups but not the 2VO-noflx group, between the latencies on the 2-choice and 1-choice tasks ($r=.85$, $p<.01$; $r=.67$, $p<.05$ and $r=.49$, $p<.40$, respectively) (Figure 6). Surprisingly, neither radial maze error type was significantly correlated with either of the water maze task latencies for any group ($p>$ at least .13).

Hippocampal Pathology

Pyramidal Cell Counts

Dorsal hippocampal tissue sections were stained with the Palmgren's silver stain. Total and damaged CA1 and CA4 pyramidal cells were counted bilaterally. Results were analyzed using a mixed analysis of variance design with group and side (left or right) as factors. Where significance was found, Scheffé's *post-hoc* test was applied to ascertain where the difference lay. The groups did not

Figure 6. Within behavioural tasks correlations. * denotes significant correlation ($p < \text{at least } .05$). **Top Panel:** Scatterplot of unbaited arm entries and baited arm re-entries on the radial maze. Data points represent individual animal's average error rates during post-surgical weeks 14, 15, and 16. **Bottom Panel:** Scatterplot of platform location latencies on the 2- and 1- cue task.

differ in the total number of cells in the CA1 ($F(2,22) < 1$, n.s.) or CA4 sectors ($F(2,23) < 1$, n.s.). Fewer cells were generally present on the right side than the left for both the CA1 ($F(1,22) = 8.22$, $p < .01$; 497 ± 20 and 542 ± 12 , respectively), and CA4 ($F(1,23) = 14.38$, $p < .001$; 94 ± 3 and 107 ± 2 respectively) sectors (Table 1).

Similar analysis of the number of damaged cells failed to reveal any group differences in either sector ($F(2,22) < 1$, n.s., for both CA1 and CA4). Unlike the total number of cells, generally there were not more damaged cells on either side of the CA1 ($F(1,22) < 1$, n.s.) or CA4 ($F(1,23) = 1.77$, n.s.) sectors.

GFAP Immunohistochemistry

Hippocampal GFAP was quantified as percent of area positively stained in the CA1 and CA4 oriens layers (Table 1). Data analysis similar to that conducted for the hippocampal cell counts found that the groups did not differ in the percent area stained in either the CA1 ($F(2,23) = 2.05$, n.s.), or CA4 ($F(2,23) = 1.52$, n.s.) sectors. Generally, the right side had a higher percent area staining than the left in the CA4 oriens layer ($F(1,23) = 9.87$, $p < .01$; $6.79 \pm .46\%$ and $5.51 \pm .36\%$ respectively), but not the CA1 oriens layer.

Hippocampal Pathology - Correlational Analysis

Averages of left and right hippocampal measures were taken as summary statistics. A negative correlation was found between the total number of CA1 pyramidal cells and CA1 GFAP staining in the 2VO-noflx group ($r = -.86$, $p < .02$) (Figure 7). This correlation relied

Table 1: Number of Pyramidal Cells and GFAP Immunoreactivity in the CA1 and CA4 Sectors of the Hippocampus

	LEFT			RIGHT		
	2VO- noflx	2VO-flx	CON	2VO- noflx	2VO-flx	CON
<i>CA1</i>						
Total Cells †	541±15	533±30	549±16	472±57	496±38	515±17
Damaged Cells	47±22	61±23	63±22	102±36	49±17	38±12
%Area GFAP	8.2±1.3	6.2±.6	5.1±.5	8.5±2.1	7.4±1.7	5.8±.4
<i>CA4</i>						
Total Cells †	107±4	110±6	104±2	96±3	90±9	96±2
Damaged Cells	10±2	12±6	10±2	11±2	6±2	8±1
% Area GFAP \$	6.3±.7	6.1±.6	4.3±.4	7.4±.9	6.8±.6	6.4±.9

† represents left > right side, $p < .01$
 ‡ represents left > right side, $p < .001$
 \$ represents left < right side, $p < .01$
 Values expressed as mean ± S.E.M.

strongly on the presence of an outlier, whose removal from the analysis resulted in the correlation losing its statistical significance. Both the 2VO-flx and CON groups did not show this correlation ($r=.03$ and $r=.36$, n.s., respectively). This relationship was not seen in the CA4 sector for any group ($r=-.23$, $r=-.27$, $r=-.40$, all n.s. for the CON, 2VO-flx and 2VO-noflx respectively), nor was the number of sick cells correlated with GFAP staining in either sector for any group ($p > \text{at least } .08$).

Visual System Pathology

Optic Tract - Gallyas Silver Staining and GFAP Immunostaining

To visualize fibre degeneration and reactive astrogliosis in the optic tract, brain sections were stained with Gallyas silver and GFAP monoclonal antibody, respectively (Figure 8). Like the hippocampal GFAP quantification, percent area of the optic tract stained was measured. Analysis of those findings revealed a significant group effect for both the Gallyas ($F(2,23) = 83.17$, $p < .001$) and GFAP stains ($F(2,23) = 44.79$, $p < .001$) (Figure 9). With both stains the 2VO-noflx group had higher percent area staining than the CON and 2VO-flx groups ($p < .01$).

Retina - Cell Layer Measurements and GFAP Immunostaining

For morphometric measurement of retinal pathology, cross-sections of retina were stained with hematoxylin and eosin (Figure 10). The thickness of the different layers, and the ganglion cell layer cell density were measured at the central nasal, central medial

Figure 7: Correlation between total hippocampal CA1 cell number and percent area GFAP immunostaining in the CA1 oriens layer. * denotes significant correlation ($p < .05$). Correlation lost significance for 2VO-noflx group when outlier was removed.

Figure 8: Photomicrographs of the right optic tract for the 2VO-noflx (A, D), 2VO-flx (B, E), and CON (C, F) groups stained with the Gallyas silver stain (A, B, C) and immunohistochemically with monoclonal anti-GFAP antibody (D, E, F). Note increased silver deposits and GFAP immunoreactivity in the 2VO-noflx group relative to the 2VO-flx and CON groups (A, D versus B, C and E, F). Scale bar reflects 100 μ m.

Figure 9: Optic tract pathology. Open and lined bars represent left and right optic tract respectively. ** denotes significantly elevated values relative to both the 2VO-flx and CON groups ($p < .01$, Scheffé's test). Error bars represent S.E.M. **Left Panel:** Percent area Gallyas silver deposits. A significant group difference was found ($F(2,23) = 83.17$, $p < .001$). **Right Panel:** Percent area GFAP immunoreactive. Again, groups differed ($F(2,23) = 44.79$, $p < .001$).

and central temporal aspects. Each was analyzed with a 3(group) by 3(aspect) mixed design analysis of variance followed by Scheffé's post-hoc test where necessary. A significant group effect was found for ganglion cell layer cell density ($F(2,23) = 10.25$, $p < .001$), and thickness of the outer plexiform $F(2,23) = 9.20$, $p < .001$, and outer nuclear ($F(2,23) = 9.03$, $p < .001$) layers (Table 2). In all three measures the 2VO-noflx group had more severe retinal pathology than either the 2VO-flx or CON groups ($p < .05$).

Ganglion cell layer cell density, as well as inner plexiform, inner nuclear and outer nuclear layer thicknesses were different at different aspects ($F(2,46) = 19.02$, $p < .001$; $F(2,46) = 36.76$, $p < .001$; $F(2,46) = 12.72$, $p < .05$; $F(2,46) = 3.31$, $p < .05$, respectively). Cell density in the ganglion cell layer and layer thickness for all but the outer nuclear layer was greater in the central medial aspect relative to the central nasal and central temporal aspects ($p < .01$). The central medial outer nuclear layer only differed from the central temporal aspect ($p < .05$).

In order to investigate whether retinal pathology was ongoing, GFAP immunohistochemistry on retinal sections was completed (Figure 10). For each aspect previously examined, GFAP staining in each cell layer was rated by an experimenter kept blind to the group membership. A 0 to 4 ordinal scale was used where 0 represented no visible staining, and 4 representing extensive staining (Table 3). Differences between the 2VO-noflx and CON groups and between the 2VO-noflx and 2VO-flx groups were analyzed with Mann-Whitney U-tests, adjusting alpha to .025 to keep the familywise error rate low. No

Table 2: Retinal Layer Thickness and Ganglion Cell Layer Cell Density

Retinal Measure	Aspect		CON	2VO-flx	2VO-noflx
RGC Density (#/mm)	Medial		60.0±5.9	65.5±5.3	42.3±3.9
	Nasal	††	35.4±4.6	47.2±4.3	27.1±3.2
	Temporal	††	47.0±2.2	46.1±5.4	24.7±5.7
			*	**	
IPL Thickness (µm)	Medial		31.3±0.9	31.4±2.0	25.5±2.8
	Nasal	††	22.7±1.3	26.3±1.8	19.0±2.5
	Temporal	††	25.3±2.1	24.4±2.4	18.6±3.4
INL Thickness (µm)	Medial		15.7±0.7	16.8±1.2	17.8±1.5
	Nasal	††	11.4±0.7	13.2±0.8	15.3±2.2
	Temporal	††	14.4±1.1	13.5±1.1	12.4±1.0
OPL Thickness (µm)	Medial		5.6±0.4	4.9±0.6	2.4±1.1
	Nasal		5.1±0.6	5.4±0.5	2.3±1.1
	Temporal		5.8±0.5	5.7±0.4	2.8±1.0
			**	**	
ONL Thickness (µm)	Medial		21.2±2.3	20.7±2.9	6.4±3.6
	Nasal		22.8±2.2	22.8±2.3	8.1±4.2
	Temporal	†	25.7±1.6	22.0±3.2	10.0±4.6
			**	**	

* represents group differing from the 2VO-noflx group, $p < .05$

** represents group differing from 2VO-noflx group, $p < .01$

† represents aspect differing from medial aspect, $p < .05$

†† represents aspect differing from medial aspect, $p < .01$

Values represent mean ± S.E.M.

Figure 10: Photomicrographs of the central medial retina for the 2VO-noflx (A, D), 2VO-flx (B, E), and CON (C,F) groups stained with hematoxylin and eosin (A, B, C) and immunohistochemically with monoclonal anti-GFAP antibody (D, E, F). Note thinned or missing outer nuclear layer and outer plexiform layer, as well as fewer retinal ganglion cell layer nuclei in the 2VO-noflx group (A) relative to the 2VO-flx (B) and CON (C) groups. No difference was seen in GFAP immunostaining between groups (B, C, D). Scale bar represents 100 μ m.

Table 3: Glial Fibrillary Acidic Protein Ratings in Different Layers of the Retina

Group	Score	N F L	G C L	I P L	I N L	O P L	O N L
		<u>m n t</u>	<u>m n t</u>	<u>m n t</u>	<u>m n t</u>	<u>m n t</u>	<u>m n t</u>
	0	- - -	- 2 -	2 2 3	4 4 4	6 6 5	6 6 5
2VO-	1	- - -	2 - 2	2 3 1	1 - -	- - -	- - -
noflx	2	1 2 1	1 1 2	1 - -	- 1 1	- - -	- - -
(n=6)	3	4 2 3	1 2 -	1 1 1	1 1 1	- - -	- - -
	4	1 2 1	2 1 1	- - -	- - -	- - -	- - -
	0	- - -	2 5 2	8 8 8	8 8 9	8 9 9	9 9 9
2VO-	1	1 4 2	5 3 4	- 1 -	1 1 -	1 - -	- - -
flx	2	5 3 3	1 - 2	1 - 1	- - -	- - -	- - -
(n=9)	3	2 1 3	1 1 -	- - -	- - -	- - -	- - -
	4	1 1 1	- - 1	- - -	- - -	- - -	- - -
	0	- - -	1 5 2	7 9 9	7 10 9	9 10 10	10 10 10
CON	1	2 2 4	5 5 7	1 1 1	2 - 1	1 - -	- - -
(n=10)	2	2 4 2	1 - 1	1 - -	1 - -	- - -	- - -
	3	4 4 4	3 - -	1 - -	- - -	- - -	- - -
	4	1 - -	- - -	- - -	- - -	- - -	- - -

Values represent the number of cases with each score

Score of 0 represents no GFAP immunoreactivity

Score of 4 represents extensive immunoreactivity

NFL represents nerve fibre layer

GCL represents ganglion cell layer

IPL represents inner plexiform layer

INL represents inner nuclear layer

OPL represents outer plexiform layer

ONL represents outer nuclear layer

m represents central medial retina

n represents central nasal retina

l represents central temporal retina

No significant group differences noted in any retinal layer ($p > .05$)

differences were noted in any retinal layer.

Visual System Pathology - Correlational Analysis

Left and right percent area of the optic tract staining for GFAP were averaged, as were those for the Gallyas silver stain. Optic tract Gallyas and GFAP silver deposits were not significantly correlated in the CON or 2VO-noflx groups. A significant positive correlation between those measures for the 2VO-flx group ($r=.83$, $p<.01$), was likely the function of 3 animals in that group exhibiting more silver and GFAP than the remaining 6 (Figure 12).

Retinal measures from the central medial aspect which significantly differed were also subjected to correlational analysis. Outer plexiform and outer nuclear layer thicknesses were significantly positively correlated for the 2VO-flx, 2VO-noflx groups, but narrowly missed significance for the CON group ($r=.70$, $p<.05$; $r=.87$, $p<.01$ and $r=.62$, $p<.06$, respectively) (Figure 12). This was not surprising as fibres originating from photoreceptors in the outer nuclear layer comprise a large part of the outer plexiform layer. The degree of ganglion cell layer loss was not correlated with either outer nuclear or outer plexiform layer thickness for any group ($p<$ at least .13 for all groups), suggesting the causes of putative ganglion cell and photoreceptor loss were not closely related.

As the optic tract consists of axons originating from the RGCs, optic tract degeneration and astrogliosis was predicted to be correlated with ganglion layer cell loss. However, no significant

Figure 11: Visual system pathology correlations. * denotes significance of correlation ($p < .05$). **Top Panel:** Relationship between percent area optic tract Gallyas silver deposits and percent area optic tract GFAP immunoreactive. **Bottom Panel:** Relationship between retinal outer nuclear layer thickness and outer plexiform layer thickness.

correlation was found for any group ($p >$ at least .21 for all correlations).

Generalized Brain Pathology - Correlational Analysis

In order to ascertain if within each group animals with greater hippocampal pathology also had greater optic tract pathology, correlational analysis was performed. The total number of hippocampal CA1 pyramidal cells was negatively correlated with Gallyas silver staining in the optic tract for the 2VO-noflx group ($r = -.82$, $p < .03$), though when an outlier was pulled from the analysis significance was lost (Figure 12). Similarly, a significant negative correlation between CA1 and optic tract GFAP staining for that group ($r = -.77$, $p < .04$) lost its statistical significance once outliers were removed (Figure 12). No other significant correlations between the measures mentioned above were found for any group ($p >$ at least .07).

Pathology and Behaviour - Correlational Analysis

Radial Arm Maze

Averages of BAR and UAE daily error rates across post-surgical weeks 14, 15, 16 were calculated and correlated with measures of hippocampal and visual system pathology. A correlation in the 2VO-flx group between total number of CA1 pyramidal cells and BAR errors ($r = -.82$, $p < .02$) (Figure 13) was noted, though the removal of an outlier resulted in the loss of significance. Correlations were not noted between these variables for the CON or 2VO-noflx groups ($r = -.07$, $p < .85$ and $r = .67$, $p < .11$, respectively). A similar relationship between

Figure 12: Correlations between hippocampal and optic tract pathologies. * denotes significance of correlation ($p < 0.05$). **Top Panel:** Relationship between the total number of CA1 pyramidal cells and percent area optic tract Gallyas silver deposits. Significance of correlation was lost for the 2VO-noflx group when an outlier was removed from the analysis. **Bottom Panel:** Relationship between CA1 and optic tract percent area GFAP immunoreactive. Significance of correlation was lost for the 2VO-noflx group when an outlier was removed from the analysis.

Figure 13: Correlations between hippocampal pathology and errors on the radial maze. * denotes significance of correlation ($p < 0.05$). **Top Panel:** Relationship between the total number of CA1 pyramidal cells and baited arm re-entries. Significance of correlation was lost for the 2VO-flx group when an outlier was removed from the analysis. **Bottom Panel:** Relationship between percent area of the CA1 sector immunoreactive for GFAP and unbaited arm entries. Significance of correlation was lost for the 2VO-noflx group when an outlier was removed from the analysis.

CA1 cells and UAE errors narrowly missed significance for the 2VO-flx, and 2VO-noflx groups ($r=-.70$, $p<.06$ and $r=-.71$, $p<.07$). Neither relationship was seen in the CON group ($r<|.41|$, $p<.26$).

The more CA1 sector hippocampal GFAP staining the more UAE errors were committed by the 2VO-noflx group ($r=.83$, $p<.02$) (Figure 14), though removal of an outlier resulted in the loss of statistical significance. This relationship was not present for the CON or 2VO-flx groups ($r=.17$, $p<.64$ and $r=.01$, $p<.98$ respectively). CA1 GFAP was not significantly correlated with BAR errors for any group ($r=.53$, $p<.11$; $r=.36$, $p<.35$ for CON and 2VO-flx respectively), though that with the 2VO-noflx group approached significance ($r=.68$, $p<.09$).

Correlations between retinal pathology and radial maze performance were investigated. Neither optic tract Gallyas stain or GFAP stain was correlated with BAR errors for any group ($p>$ at least .18). Optic tract GFAP was significantly correlated to UAE errors for the 2VO-noflx group ($r=-.91$, $p<.005$) (Figure 14). This negative correlation was surprising as it indicated that greater amounts of GFAP in the optic tract were related to better performance on the radial maze.

Ganglion cell layer cell density was not related to either BAR or UAE errors for either group ($p<.11$). Contrary to expectations, the thicker a control animal's outer nuclear layer (presumably reflecting more photoreceptors), the more UAE errors were made ($r=.76$, $p<.01$). This relationship did not exist for the 2VO-flx ($r=-.16$, $p<.68$), or 2VO-noflx ($r=.14$, $p<.77$) groups.

Figure 14: Correlations between visual system pathology and errors on the radial maze. * denotes significance of correlation ($p < .05$). **Top Panel:** Relationship between percent area of the optic tract immunoreactive for GFAP and unbaited arm entries. **Bottom Panel:** Relationship between retinal outer nuclear layer thickness and unbaited arm entries.

Vision Tasks

Possible relationships between visual system and hippocampal pathologies and vision task performance were explored by trying to relate total CA1 pyramidal cell number, CA1 GFAP staining, optic tract Gallyas and GFAP staining, ganglion cell layer cell density and outer nuclear layer thickness with the mean platform location latencies from the last two days of the 2-cue task and the last day of the 1-cue task averaged. The only significant correlation was for the CON group where optic tract Gallyas staining was negatively correlated with latency ($r = -.64$, $p < .05$) (Figure 15). It is counter-intuitive that greater optic tract pathology exists along with better vision task performance. All other correlations did not approach significance ($p >$ at least .24).

Figure 15: Correlation between percent area of optic tract with Gallyas silver deposits and the average vision task latency. * denotes significance of correlation ($p < \text{at least } .05$).

DISCUSSION

After 2VO, 7 of 16 rats (44%) lost the pupillary reflex. Despite this indication of visual system pathology in the 2VO-noflx group, subsequent testing on a radial maze task revealed consistently higher rates of UAE and BAR errors only after 6 and 11 weeks of occlusion, respectively. 2VO rats who retained the reflex did not show increased UAE or BAR errors. Performance on explicitly visual tasks after approximately 30 weeks of ligation was significantly impaired only for the 2VO-noflx group.

After approximately 41 weeks of 2VO the groups did not differ in the total damaged hippocampal CA1 or CA4 pyramidal cells. Furthermore, the groups did not have different levels of GFAP immunoreactivity in CA1 or CA4 or in the retina. However, the outer nuclear and outer plexiform layers of the retina were thinner in the 2VO-noflx group relative to the 2VO-flx and CON groups. As well their ganglion cell layer cell density was lower.

Loss of Pupillary Reflex

We became aware that the visual system may be affected by 2VO while the experiment was already under way. As a result, only 12 animals had their reflex observed from surgery onwards. Four of the 8 2VO rats lost their pupillary reflex within 1 week post-ligation. Of the animals whose pupillary reflexes were monitored from approximately 16 weeks post-surgically, 3 of 8 2VO animals had lost the reflex. As the 2VO animals who were monitored from surgery lost

their reflex very soon following ligation, and no animals lost or regained the reflex at later post-surgical times, those animals whose reflex was first inspected at the end of radial maze testing were grouped together with animals who were observed to have lost the reflex shortly after surgery. Ongoing studies in this laboratory also indicate that the permanent loss of pupillary reflex occurs within 10 days of 2VO (Bennett et al., in preparation; Pappas et al., in preparation).

Many factors may cause the loss of pupillary reflex. Dysfunction of the retina, olivary pre-optic nucleus, ventral lateral geniculate, Edinger-Westphal nucleus, ciliary ganglion, or any of the fibres which connect these structures may result in the loss of pupillary reflex. It is also possible that excessive sympathetic stimulation from the superior cervical ganglion may also lead to unresponsive pupils. However, the most obvious explanation for the loss of reflex in 2VO rats is that their optic tract degeneration and/or retinal ganglion cell death effectively prevented retinal signals from reaching the brain centres which mediate the reflex. The findings that optic tract pathology is present by 1 week of 2VO supports this idea (Wakita et al., 1994). However, it is unclear if this pathology is sufficient to cause the loss of the reflex. Electrophysiological studies are required to establish if the optic nerve/optic tract of 2VO rats is able to transmit signals to reflex-associated brain centres.

It needs to be determined why only some 2VO animals lose the pupillary reflex. There is variability among rats in the structure

of the circle of Willis (Scremin, 1995). Perhaps animals whose arterial circle permitted less perfusion were subject to a greater degree of retinal ischemia resulting in damage to RGCs and/or other components of the pupillary reflex circuit.

Behavioural Effects

All animals were trained prior to surgery to a pre-set criteria on an 10-arm radial maze, with 2 of the arms never containing a food reward. Entries to these two arms comprised unbaited arm entries, while entries into arms from which the animal had already retrieved the food reward comprised baited arm re-entries.

Overall, the 2VO-noflx but not the 2VO-flx group made more BAR and UAE errors than the CON group after surgery. The 2VO-noflx group also generally made more UAE errors than the 2VO-flx group. They consistently made more UAE errors after 6 weeks of occlusion and more BAR errors after 10 weeks. If UAEs and BARs reflect reference and working memory errors respectively (Olton and Papas, 1979), then reference memory deficits emerged before working memory deficits .

Our laboratory has previously found that 2VO results in more UAEs after 7 weeks of occlusion (Pappas et al., 1996b), very similar to what was seen here. Pupillary reflex was not recorded in that study. Perhaps in that study a subgroup of 2VO animals without pupillary reflex started to perform more poorly after 7 weeks, raising the overall group UAE error mean. Increased BAR errors after 2VO were not seen, although the 2VO group mean was consistently higher than the CON mean at all time points examined. Conceivably,

a subgroup of animals lacking the pupillary reflex may have caused the higher error rates, raising the overall group mean insufficiently for statistical significance.

In the present study, BAR and UAE errors were correlated for the CON and 2VO-flx groups, but not for the 2VO-noflx group. If in fact vision deficits occurred in this group, these animals may have solved the radial maze using a non-visual strategy (such as using a simple alternating algorithm) where arm re-entries (and thus BAR errors) are minimized, but which cannot prevent the first entrance into an unbaited arm (and thus UAE errors). This would explain why BAR and UAE errors were not correlated for the 2VO no-flx animals.

Visual system dysfunction was assessed in the water maze set up as a 2-cue, then a 1-cue task. The 2-cue task had a black and a yellow visual cue located randomly at one of two places on the maze. The submerged (not visible) platform was always located under the black platform. The 1-cue task was similar, but only had the black cue. The black cue (and platform) was randomly placed around the maze to minimize the effectiveness of any non-visual search strategy.

The 2VO-noflx animals had longer latencies on both the 2-cue and 1-cue tasks than the 2VO-flx and CON groups, suggesting that they had more difficulty locating the correct cue. This was not a function of different swim speed as they did not vary between groups. Furthermore, 2VO-noflx animals did not first swim under the correct cue more often than chance, while both the 2VO-flx and CON groups did. These two findings suggest that the 2VO-noflx animals had a vision deficit.

While it is feasible that the 2VO-noflx animals could not learn that swimming towards the black cue would lead to finding the platform, such a severe deficit seems unlikely in view of the limited brain pathology discussed below. As well, current research in this laboratory indicates that 2VO-noflx rats lose the ability to perform visual, but not tactile discrimination.

Other studies involving 2VO have also found that animals could perform only at a chance level on visual discrimination tasks at 1 to 30 days, and 6 weeks post-ligation (Yamamuro et al., 1996; Tanaka et al., 1996). Our findings extend this time point to approximately 30 weeks post-ligation.

Error rates on the radial maze did not correlate with latencies or percent correct annulus crossings in the water tasks. It should be noted that since the 2VO-noflx group performed more poorly on both the radial maze and vision tasks, performance on these tasks may be related despite the lack of a significant correlation.

Pathology

Hippocampal pathology was assessed using Palmgren's silver stain, and GFAP immunostaining. The total and number of damaged pyramidal cells in the CA1 and CA4 sectors were counted at 3 different distances from bregma. Percent areas immunoreactive for GFAP in the oriens layers of these sectors were also measured. Unlike other studies which found CA1 cell loss after 3, 4 and approximately 6 months after 2VO (Ohta et al., 1997; Ni et al., 1994, Ni et al., 1995; Pappas et al., 1996b), neither the 2VO-flx or 2VO-

noflx groups exhibited CA1 cell loss relative to controls after approximately 9.5 months. Similarly, unlike a previous report of GFAP increases in the CA1 oriens layer following approximately 6 months of 2VO (Pappas et al., 1996b), no increases were noted. It is possible that cells normally destined to die as a result of natural aging processes, die sooner as a result of 2VO. Perhaps, at approximately 22 months of age, both CON and 2VO animals have lost these pyramidal cells, and thus have a similar number of cells.

It is possible that there were fewer hippocampal CA1 pyramidal cells in the 2VO animals, but greater tissue shrinkage in those animals may have obscured this finding. The greater the degree of shrinkage, the more neurons would be present in any given brain slice. This also casts a shadow on previous published findings of reduced cell number and GFAP immunoreactivity increases, as the effect of tissue shrinkage was not addressed. Optical fractionation is a technique which controls for this, and yields estimates of total cells per structure (Wickelgren, 1996). Future experiments should adopt this technique of cell counting.

Interestingly, there were fewer CA1 and CA4 pyramidal cells and greater CA4 sector GFAP immunoreactivity in the right hippocampus compared to the left, across groups. To our knowledge, no other study has described this asymmetry. Since this was not seen preferentially in any group, it may reflect a sampling bias in data collection, though this is unlikely as the same sampling criteria were applied to both the left and right hemispheres. Hippocampal CA1 and CA4 total cells, and hippocampal GFAP initially appeared to be

correlated for some groups, however the removal of an outlier made the correlations no longer statistically significant.

Optic tract GFAP and Gallyas silver staining was elevated in the 2VO-noflx group relative to the CON or 2VO-flx groups, indicating greater glial infiltration and more damaged axons after approximately 41 weeks of ligation in animals without the pupillary reflex. This adds to published research showing that optic tract degeneration is evident after 1 and 16 weeks of 2VO (Takamasu, et al., 1984; Wakita et al., 1994; Ohta et al., 1997).

The cause of this degeneration is not known. It is possible that chronic ischemia results in RGC death, and that their axons subsequently degenerate. It is also possible that 2VO results in ischemia of the optic nerve sufficient to cause white matter rarefaction, and subsequent death of RGCs. Yet another possibility is trans-synaptic degeneration from brain structures receiving retinal input such as the dorsal and ventral thalamus, pretectum and superior colliculus. Perhaps these areas first succumb to ischemia and there is subsequent retrograde, transynaptic degeneration of the optic tract, optic nerve and RGCs.

Gallyas staining and GFAP immunostaining in the optic tract were significantly positively correlated for the 2VO-flx group. This was likely a reflection of 3 animals in that group who demonstrated both more Gallyas and GFAP staining. There was no correlation for the other two groups.

Curiously, the number of nuclei in the ganglion cell layer did not correlate with the degree of optic tract GFAP infiltration nor

the amount of Gallyas staining. For the 2VO-flx and CON groups it is possible that since there was little or no degeneration in the optic tract or ganglion cell layer these measures of pathology are not correlated. For the 2VO-noflx group which exhibited fewer RGC cell layer nuclei, and more optic tract pathology, the lack of correlation is surprising. There are several possible explanations. Firstly, the RGC cell layer is comprised not only of retinal ganglion cells, but also displaced amacrine cells. The number of amacrine cells would not be expected to be as closely related to optic tract patency as is the number of RGCs. Thus, the amacrine cell loss may blur the correlation between RGC number and optic tract pathology. It is also possible that the temporal profile of RGC death optic nerve damage, and astrocyte hypertrophy may differ. All three measures may be related, but when examined at one time point there may not be a significant correlation.

The photoreceptor containing outer nuclear layer, and the outer plexiform layer containing associated fibres were thinner for the 2VO-noflx group relative to the 2VO-flx and CON groups. This may be a result of the lack of pupillary reflex rendering the retina relatively defenceless against ambient laboratory light. Animals were kept on a 12 hour day light cycle, which may prove toxic to photoreceptors which are not protected by a pupillary reflex. Another possibility may be that photoreceptors are very sensitive to reductions in blood flow. To our knowledge no reports exist of high sensitivity of photoreceptors to ischemia. As well, photoreceptor death probably was not a result of RGC death. It seems unlikely that

RGC death would result in photoreceptor and not bipolar cell death.

GFAP immunoreactivity in the retina did not differ between groups and was mostly noted in the ganglion cell and nerve fibre layers. Previous research has shown that optic nerve crush, transection, and penetrating eye wounds result in the appearance of GFAP staining along Müller cell processes in all cell layers (Huxlin et al., 1995; Bignami et al., 1979). The GFAP increases following optic nerve transection were shown to abate 14 days after injury, suggesting that GFAP increases are a transient response to injury. If this is the case, one may conclude that the pathological changes in the retina are fully elaborated sometime before 41 weeks in the 2VO-noflx animals.

To determine whether or not 2VO results in generalized pathology in both the optic tract and the hippocampus correlational analysis between measures of pathology in these areas was carried out. A significant negative correlation for the 2VO-flx group between the total number of CA1 pyramidal cells and the percent area of Gallyas silver staining in the optic tract seemed to contradict a significant negative correlation between GFAP staining in the CA1 sector and in the optic tract. The former suggested that greater optic tract pathology was related to less hippocampal pathology, and the latter supported co-varying degrees of damage in those structures. Removal of an outlier from the correlational analysis resulted in the loss of statistical significance in both correlations.

Further evidence that optic tract and hippocampal pathologies were not related comes from the finding that the 2VO-noflx group had

greater optic tract pathology than the 2VO-flx group, yet these groups did not differ on measures of hippocampal pathology. If pathology in these two areas were related, one would expect to see group differences in the pathology of both areas.

Correlations of Pathology and Behaviour

Correlational analysis was conducted to relate measures of pathology to performance on the behavioural tasks. While significant correlations between hippocampal pathology and radial maze performance were found for both the 2VO-flx, and 2VO-noflx groups, these were found to be not significant when an outlier was removed from the analysis. In each of these groups, one animal with greater hippocampal pathology made considerably more errors on the radial maze for each group. Thus, a general correlation between radial maze performance and hippocampal pathology was not found. It must be kept in mind that the time difference between hippocampal pathology measures and measures of radial maze performance was approximately 28 weeks, so the relationship between hippocampal viability when the rats were running the maze and maze performance remains unknown.

Two perplexing correlations were found between measures of visual system pathology and radial maze performance. Optic tract GFAP was negatively correlated with UAE errors in the radial maze for the 2VO-noflx group, suggesting greater optic tract pathology is related to better performance on this maze. Perhaps activated astrocytes, while being a marker of pathology, play a role in preserving optic tract function. Thus more astrocytes may represent

less compromised vision.

For the CON group, outer nuclear layer thickness was positively correlated with UAE errors, suggesting animals with more photoreceptors make more UAE errors on the radial maze. This finding is counter-intuitive and we have no explanation.

A similarly perplexing correlation was found for the control group between optic tract Gallyas staining and platform location latency on visual tasks. Animals with greater optic tract pathology took less time to find the platform.

Assuming that the rats solve the radial maze using visually-guided strategies, it is perplexing that UAE and BAR errors were not consistently higher for 2VO-noflx animals until after week 6 and week 10 respectively. The loss of pupillary reflex in this group shortly after surgery suggests that vision is compromised to some degree shortly after occlusion. It is possible that vision was not compromised to a sufficient degree so as to result in group differences until these later time points. For that matter, it is possible that vision was not seriously affected at any time after ligation and the group differences were a function of pathology in other areas of the brain. However, this is unlikely as other studies suggest that vision maybe be disrupted shortly after ligation. Electroretinograph b-waves have disappeared in 2VO animals after 1 week of occlusion (Barnett and Osborne, 1995) suggesting that signal transmission in the retina is reduced or gone. Optic tract rarefaction was seen after 1 week of ligation (Wakita et al., 1994). Behaviourally, 2VO animals perform only at a chance level on a visual

discrimination task when tested shortly after surgery (Yamamuro et al., 1996).

It is possible that the maze was solved using a non-visual strategy, and that the neural structures underlying this strategy were subject to delayed damage. Although not replicated here, hippocampal CA1 cell loss has been reported after 4 months and approximately 27 weeks (~6.3 months) of ligation, but not after 2 weeks or 1 month (Ni et al., 1994; Ni et al., 1995; Pappas et al., 1996b). Furthermore, reductions in muscarinic acetylcholine receptors in the hippocampus, frontal cortex, and striatum have been seen after 6 weeks of ligation (Tanaka et al., 1996; Kondo et al., 1996). These changes or other delayed changes may underlie late emerging radial maze deficits despite vision deficits.

Clinical Relevance of the Current Findings

In human, the retina depends less on the external ophthalmic artery and receives most of its arterial blood supply from the human analog to the rat's internal ophthalmic artery, which originates from the internal carotid at the base of the brain (Scremin et al., 1995). Complete or partial stenosis of the common or internal carotid results in retinal blood flow reductions. Carotid stenosis often results in amourosis fugax, or if sufficiently severe and chronic, neovascular glaucoma (Breen, 1991; Dugan and Green, 1991). Thus, reduction of retinal blood flow in both rats with 2VO and humans with carotid stenosis result in vision deficits, suggesting 2VO may be a good model for studying the effect of carotid stenosis on the visual

system.

In the past few years vision deficits have been investigated in Alzheimer's patients. Among the findings reported are retinal ganglion cell loss (Blanks et al., 1989), reductions in visual acuity (Sadun et al., 1987), poorer ability to differentiate blue hues (Cronin-Golomb et al., 1993), b-wave reduction in the pattern-induced electroretinogram (Katz et al., 1989) and optic nerve pathology (Hinton et al., 1986). Unlike cerebral pathology, no amyloid plaques or neurofibrillary tangles have been seen in the retinas of Alzheimer's patients. Cerebral blood flow is reduced in Alzheimer's disease (Prohovnik, 1988; Rogers et al. 1986; Deutsch and Tweedy, 1987), though whether flow to the retina is reduced has not been established. If retinal blood flow reductions are related to vision deficits in AD result 2VO may be suitable animal model for this as well.

Finally, it should be noted that 2VO resulted in a late emerging deficit on the radial maze in some animals, which may reflect memory deficits, despite the animals having vision deficits. Perhaps the later emerging cholinergic deficits seen by others and/or recently described energy metabolism deficits (reduced cytochrome oxidase activity) (de la Torre et al., 1997) underlie memory deficits. These changes may be functionally important, but not evident by cell counts and GFAP immunoreactivity. If so, then 2VO may yield insights into the role of reduced CBF in the memory deficits seen in Alzheimer's disease.

Suggestions for Further Studies

Future studies should examine the biological substrates underlying the loss of pupillary reflex, and poorer performance on the visual tasks. Firstly, retino-receptient brain centres should be examined on the tissue remaining from this study. Preliminary research in this laboratory suggest that GFAP is increased in the pupillary reflex associated ventral lateral geniculate nucleus after 27 to 40 weeks post-surgically. This could be followed by a time course study, documenting the development of visual system pathology in the different retinal, white matter and brain centres.

Further investigations of the effect of reduced CBF on spatial learning should be assessed by chronic occlusion of the internal carotid arteries superior to the pterygopalatine artery. This would largely remove the confound of reduced blood flow to the retina (Ohta et al., 1997).

The extensive body of literature which relates transient ischemia-induced CA1 pyramidal cell loss with deficits in spatial maze tasks has not considered the effect of ischemia on the visual system. It is entirely possible that visual system pathology is the causative factor behind spatial task deficits and could explain poor correlations between hippocampal cell loss and spatial memory ability (Nunn et al., 1994; Olsen et al., 1994). Vision should be assessed in these models of transient ischemia.

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APPENDIX A.1
ANOVA Summary Table for Baited Arm Re-entry Errors

	SS	df	MS	F	p
GROUP	75.29	2	37.64	6.44	.007
ERROR	116.83	20	5.84		
WEEK	10.65	15	.71	.95	.511
GROUP BY WEEK	14.93	30	.50	.67	.907
ERROR	223.32	300	.74		

APPENDIX A.2
Summary of Separate ANOVAs for Each Week's
Mean Daily Unbaited Arm Entry Errors

Week	df	MSgroup	MSerror	F	p
1	2,23	4.99	1.71	2.92	.074
2	2,24	5.82	1.05	5.53	.011
3	2,23	1.78	.67	2.65	.092
4	2,22	.66	.48	1.38	.271
5	2,24	2.59	1.89	1.32	.272
6	2,24	3.06	.50	6.12	.007
7	2,24	2.95	.93	3.18	.059
8	2,24	1.74	1.09	1.60	.222
9	2,24	4.75	1.44	3.29	.055
10	2,24	6.47	1.34	4.82	.017
11	2,24	8.77	2.07	4.24	.026
12	2,24	3.20	.86	3.74	.039
13	2,24	4.77	1.37	3.49	.047
14	2,24	2.90	.72	4.05	.030
15	2,24	11.24	1.45	7.76	.003
16	2,24	5.84	2.03	2.87	.007
23	2,20	10.74	1.47	7.28	.004

APPENDIX A.3

ANOVA Summary Table for the Effect of a 7-Week
Break in Radial Maze Testing on Baited Arm Re-Entry Errors

	SS	df	MS	F	p
GROUP	23.68	2	11.84	6.47	.007
ERROR	34.75	19	1.83		
PRE_POST	7.40	1	7.4	4.88	.040
GROUP BY PRE_POST	3.01	2	1.51	.99	.389
ERROR	28.85	19	1.52		

APPENDIX A.4

ANOVA Summary Table for Unbaited Arm Entry Errors

	SS	df	MS	F	p
GROUP	21.55	2	10.78	6.02	.009
ERROR	35.81	20	1.79		
WEEK	8.09	15	.54	3.02	.000
GROUP BY WEEK	6.30	30	.21	1.17	.248
ERROR	54.64	300	.18		

APPENDIX A.5
Summary of Separate ANOVAs for Each Week's
Mean Daily Baited Arm Re-Entry Errors

Week	df	MSgroup	MSerror	F	p
1	2,23	.97	.68	1.44	.258
2	2,24	.27	.27	1.02	.374
3	2,23	.83	.18	4.65	.020
4	2,22	.18	.20	.91	.416
5	2,24	.20	.35	.57	.576
6	2,24	.95	.20	4.77	.018
7	2,24	1.31	.24	5.55	.010
8	2,24	.75	.20	3.69	.040
9	2,24	1.83	.25	7.30	.003
10	2,24	1.54	.23	6.58	.005
11	2,24	2.77	.33	8.45	.002
12	2,24	1.48	.30	4.91	.016
13	2,24	2.45	.18	13.54	.000
14	2,24	1.99	.30	6.55	.005
15	2,24	2.18	.48	4.52	.022
16	2,24	2.71	.37	7.43	.003
23	2,20	2.80	.22	12.71	.000

APPENDIX A.6

ANOVA Summary Table for the Effect of a 7-Week Break in
Radial Maze Testing on Unbaited Arm Entry Errors

	SS	df	MS	F	p
GROUP	8.08	2	4.04	10.50	.001
ERROR	7.30	19	.38		
PRE_POST	1.39	1	1.39	7.86	.011
GROUP BY PRE_POST	.17	2	.09	.49	.622
ERROR	3.36	19	.18		

APPENDIX A.7
Design ANOVA Summary Table for Platform
Location Latencies on the 2-Cue Vision Task

	SS	df	MS	F	p
GROUP	15450.21	2	7525.10	25.61	.000
ERROR	6681.80	21	318.18		
DAY	20163.14	9	2240.35	58.24	.000
GROUP BY DAY	1249.79	18	69.43	1.66	.024
ERROR	7169.98	189	38.46		

APPENDIX A.8

ANOVA Summary Table for Percent Correct
First Annulus Crossings on the 2-Cue Vision Task

	SS	df	MS	F	p
GROUP	1916.59	2	958.29	1.97	1.65
ERROR	10235.64	21	487.41		
DAY	3775.95	9	419.55	1.98	.043
GROUP BY DAY	3270.18	18	181.70	.86	.629
ERROR	39978.38	189	211.53		

APPENDIX A.9
ANOVA Summary Table for Platform
Location Latencies on the 1-Cue Vision Task

	SS	df	MS	F	p
GROUP	6936.65	2	3468.52	21.88	.000
ERROR	3329.42	21	158.54		
DAY	390.07	4	97.52	3.97	.005
GROUP BY DAY	341.22	8	42.65	1.74	.102
ERROR	2063.13	84	24.56		

APPENDIX A.10
ANOVA Summary Tables for Total Number of
Hippocampal Pyramidal Cells in the CA1 and CA4 Sectors

CA1

	SS	df	MS	F	p
GROUP	5929.22	2	2964.61	.24	.791
ERROR	275778.19	22	12535.37		
LFT_RT	24587.27	1	24587.27	8.22	.009
GROUP BY LFT_RT	2994.65	2	1497.32	.50	.613
ERROR	65805.09	22	2991.14		

CA4

	SS	df	MS	F	p
GROUP	28.38	2	14.98	.04	.954
ERROR	7265.25	23	315.88		
LFT_RT	2207.41	1	2207.41	14.38	.001
GROUP BY LFT_RT	341.04	2	170.52	1.11	.346
ERROR	3530.44	23	153.48		

APPENDIX A.11
ANOVA Summary Tables for Number of Damaged
 Hippocampal Pyramidal Cells in the CA1 and CA4 Sectors

CA1

	SS	df	MS	F	p
GROUP	5009.82	2	2504.91	.59	.563
ERROR	93309.51	22	4241.34		
LFT_RT	1.45	1	1.45	.00	.986
GROUP BY LFT_RT	14199.10	2	7099.55	1.66	.213
ERROR	94211.14	22	4282.32		

CA4

	SS	df	MS	F	p
GROUP	16.55	2	8.28	.09	.912
ERROR	2052.54	23	89.24		
LFT_RT	94.95	1	94.95	1.77	.196
GROUP BY LFT_RT	88.97	2	44.48	.83	.448
ERROR	1231.97	23	53.56		

APPENDIX A.12
ANOVA Summary Tables for Percent Area
Hippocampal GFAP in the CA1 and CA4 Sectors

CA1

	SS	df	MS	F	p
GROUP	68.09	2	34.05	2.05	.151
ERROR	381.65	23	16.59		
LFT_RT	7.28	1	7.28	1.13	.299
GROUP BY LFT_RT	1.53	2	.79	.12	.886
ERROR	381.65	23	16.59		

CA4

	SS	df	MS	F	p
GROUP	19.62	2	9.80	1.52	.240
ERROR	148.42	23	6.45		
LFT_RT	21.34	1	21.34	9.87	.005
GROUP BY LFT_RT	4.57	2	2.29	1.06	.364
ERROR	49.74	23	2.16		

APPENDIX A.13
ANOVA Summary Tables for Percent Area of
Optic Tract Stained with Gallyas Silver and GFAP

Gallyas Silver Stain

	SS	df	MS	F	p
GROUP	251.78	2	125.89	83.17	.000
ERROR	34.81	23	1.51		
LFT_RT	4.18	1	4.18	3.80	.064
GROUP BY LFT_RT	7.08	2	3.54	3.22	.058
ERROR	25.28	23	1.10		

GFAP

	SS	df	MS	F	p
GROUP	9234.64	2	4617.32	44.79	.000
ERROR	2371.27	23	103.10		
LFT_RT	180.16	1	180.16	3.92	.060
GROUP BY LFT_RT	230.81	2	115.41	2.51	.103
ERROR	1057.25	23	45.97		

APPENDIX A.14
ANOVA Summary Tables for the Thickness of
Retinal Outer Nuclear and Outer Plexiform Layers

Outer Nuclear Layer

	SS	df	MS	F	p
GROUP	3212.12	2	1606.06	9.03	.001
ERROR	4092.35	23	177.93		
ASPECT	132.27	2	66.13	3.31	.046
GROUP BY ASPECT	38.71	4	9.68	.48	.747
ERROR	919.90	46	20.00		

Outer Plexiform Layer

	SS	df	MS	F	p
GROUP	131.12	2	65.56	9.20	.001
ERROR	163.93	23	7.13		
ASPECT	3.73	2	1.86	1.01	.374
GROUP BY ASPECT	2.28	4	.57	.31	.871
ERROR	85.19	46	1.85		

APPENDIX A.15
ANOVA Summary Tables for Thickness of
Retinal Inner Nuclear and Inner Plexiform Layers

Inner Nuclear Layer

	SS	df	MS	F	p
GROUP	21.96	2	10.89	.60	.558
ERROR	421.83	23	18.34		
ASPECT	196.04	2	98.02	12.72	.000
GROUP BY ASPECT	74.85	4	18.71	2.42	.061
ERROR	354.46	46	7.71		

Inner Plexiform Layer

	SS	df	MS	F	p
GROUP	538.94	2	269.47	2.92	.074
ERROR	2122.62	23	92.29		
ASPECT	776.99	2	388.49	36.76	.000
GROUP BY ASPECT	53.92	4	13.48	1.28	.293
ERROR	486.13	46	10.57		

APPENDIX A.15
ANOVA Summary Table for Cell
Density in the Retinal Ganglion Cell Layer

	SS	df	MS	F	p
GROUP	5689.10	2	2844.55	10.25	.001
ERROR	6282.88	23	277.52		
ASPECT	6316.78	2	3158.39	19.08	.000
GROUP BY ASPECT	382.70	4	95.68	.58	.680
ERROR	7615.59	46	165.56		

IMAGE EVALUATION TEST TARGET (QA-3)

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