Retinal and Optic Nerve Degeneration After Chronic Carotid Ligation

Time Course and Role of Light Exposure

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Background and Purpose—Carotid artery disease can cause chronic retinal ischemia, resulting in transient or permanent blindness, pupillary reflex dysfunction, and retinal degeneration. This experiment investigated the effects of chronic retinal ischemia in an animal model involving permanent carotid occlusion. The time course of retinal pathology and the role of light in this pathology were examined.

Methods—Sprague-Dawley rats underwent permanent bilateral occlusion of the common carotid arteries or sham surgery. Half of the animals were postsurgically housed in darkness, and half were housed in a 12-hour light/dark cycle. Animals were killed at 3, 15, and 90 days after surgery. Stereological techniques were used to count the cells of the retinal ganglion cell layer. Thy-1 immunoreactivity was assessed to specifically quantify loss of retinal ganglion cells. The thicknesses of the remaining retinal sublayers were measured. Optic nerve degeneration was quantified with the Gallyas silver staining technique.

Results—Permanent bilateral occlusion of the common carotid arteries resulted in loss of the pupillary reflex to light in 58% of rats. Eyes that lost the reflex showed (1) optic nerve degeneration at 3, 15, and 90 days after surgery; (2) a reduction of retinal ganglion cell layer neurons and Thy-1 immunoreactivity by 15 and 90 days; and (3) a severe loss of photoreceptors by 90 days when postsurgically housed in the light condition only.

Conclusions—Ischemic damage to the optic nerve caused loss of pupillary reflex and death of retinal ganglion cells in a subset of rats. Subsequently, light toxicity induced death of the photoreceptors. (Stroke. 2002;33:1107-1112.)

Key Words: carotid artery occlusion ■ hypoperfusion ■ ischemia ■ reflex, pupillary ■ retina

Ocular symptoms are a common feature of carotid artery disease. Amaurosis fugax is probably the most frequent, occurring in approximately one third of the patients.1,2 Pupillary reflex dysfunction may also occur.3–5 The visual pathology may progress to permanent blindness.

Exposure to bright light has been shown to induce amaurosis fugax in patients with carotid artery disease,6,7 suggesting that light metabolically overburdens the moderately ischemic retina.3 Light bleaches photoreceptor pigment, which must be resynthesized by an energy-dependent process, and this capacity could be compromised by retinal ischemia.8 Hence, chronic exposure to light could ultimately lead to photoreceptor degeneration in the moderately ischemic retina.

The following experiment investigated the nature and the time course of retinal and optic nerve degeneration, as well as the role of light exposure therein, in an animal model of chronic bilateral carotid artery occlusion (2VO). Immediate abnormalities of the electroretinogram and early-appearing optic nerve pathology occur after 2VO.9–11 However, a recent study of the histological effects on the retina found surprisingly late-appearing and subtle retinal degeneration.12 By 3 months, no significant degeneration was apparent. After 9 months, an overall thinning of the retina and a significant reduction of photoreceptors were noted. The status of the pupillary reflex of these rats was not stated.

We have recently reported that 2VO caused an early loss of the pupillary reflex in approximately 50% of Sprague-Dawley rats and that severe retinal degeneration was evident only in this subset of rats.13 These rats were visually impaired and after 9 months showed optic nerve degeneration, profound photoreceptor loss, and reduction of cells in the retinal ganglion cell layer (RGCL).

The etiology and time course of the degeneration of various retinal aspects accompanying loss of the pupillary reflex are unknown. A more detailed examination of these factors could provide insight into the nature of retinal pathology associated with human carotid artery disease, perhaps suggesting therapeutic strategies. In this study we examined (1) the temporal emergence of pupillary reflex loss caused by 2VO, (2) how this related to the progression of retinal and optic nerve degeneration, and (3) the contributory factors...
role of light exposure. It was predicted that a subset of 2VO rats (approximately 50%) would suffer pupillary reflex loss accompanied by retinal and optic nerve degeneration and that exposure to light would exacerbate this pathology.

Materials and Methods

Animals
Four-month-old male Sprague-Dawley rats (n = 73; Charles River Laboratories, Montreal, Quebec, Canada) were singly housed in a 12-hour reverse light cycle (on 8 PM, off 8 AM) with free access to food and water. All procedures were approved by the Animal Care Committee of Carleton University and conformed to Canadian Council on Animal Care guidelines and the Association for Research in Vision and Ophthalmology statement for use of animals in ophthalmic and vision research.

Pupillary Reflex
The pupillary reflexes of each animal were examined on arrival and also before surgery to verify normal functioning. Direct and consensual responses were assessed in both eyes. Each animal was first adapted to darkness for at least 5 minutes. One eye was then exposed to a beam of light from an otoscope to assess the direct reflex response. The otoscope was then immediately directed at the contralateral eye to assess the consensual response. Both eyes were then allowed to readapt to darkness for approximately 1 minute. This procedure was repeated with the other eye. Loss of pupillary reflex was defined as failure of the pupil to constrict after a 10-second exposure to light. Pupillary reflexes were examined each day for the first week after surgery, at least once per week thereafter, and immediately preceding perfusion.

Surgery
Animals were randomly assigned to either 2VO surgery or sham surgery. Approximately half of the animals were postsurgically housed in constant darkness. These animals were exposed only very briefly to extremely dim room illumination during pupillary reflex observation. The rats in the light condition were housed in a regular 12-hour reverse light cycle (on 8 PM, off 8 AM). Room illumination was controlled for all animals in the lab and maintained at approximately 5 lux. Groups of animals were perfused at 3 different times: either 3, 15, or 90 days after surgery.

Surgery was performed under ketamine hydrochloride (100 mg/kg IM) and methohexital sodium (40 mg/kg IP) anesthesia. Animals were also administered atropine sulfate (0.1 mg IM) to prevent respiratory distress. Their eyes were covered during surgery to prevent light exposure. A ventral midline incision was made, and the common carotid arteries were bilaterally separated from the carotid sheath and vagus nerve. Each common carotid artery was doubly ligated with 5-0 silk suture approximately 8 to 10 mm inferior to the origin of the external carotid artery. The incision was then sutured. Animals assigned to sham surgery underwent the same procedure except that the common carotid arteries were not ligated.

Tissue Preparation and Histology
Animals were deeply anesthetized with ketamine hydrochloride and methohexital sodium and then transcardially perfused with paraformaldehyde. A suture was placed in each eye to facilitate later surgery. Multiple thickness measures (approximately 120 to 160 measures) were deparaffinized and rehydrated through graded ethanol solutions and distilled water. Sections were incubated at room temperature in Mayer’s hematoxylin, placed under running warm water for 30 minutes, then dehydrated, cleared, and coverslipped.

Cell counts were performed at ×1000 magnification with the use of an Olympus BH-2 microscope connected to a Sony charge-coupled device video camera, a motorized stage system, and commercial stereology software (Stereo Investigator, MicroBrightField, Inc). The optical dissector technique was used to avoid double counting of cells.\textsuperscript{15} The sampling interval was every ninth section, sampling grid size was 120×120 μm, and counting frame size was 18×18×10 μm. These parameters yielded an acceptable coefficient of error of ≤0.08. The starting depth of the counting frame was 3 μm below the surface of the tissue, providing a guard zone to prevent counting of lost caps and neurons damaged during sectioning.

To determine whether reductions of neuron numbers in the RGCL reflected loss of retinal ganglion cells (RGCs) specifically, sections were immunohistochemically stained with a monoclonal antibody raised in mouse against Thy-1 (PharMingen International). Thy-1 expression within the retina is specific to RGC bodies and their processes.\textsuperscript{16,17}

Sections (3 per eye) adjacent to those selected for thickness measures (see below) were deparaffinized, rehydrated, then incubated overnight in mouse anti-Thy-1 (1:1000) diluted in a blocking buffer of 0.3% lambda carrageenan, 0.3% bovine serum albumin, and 0.3% Triton X-100 in PBS. Sections were then washed in PBS before being incubated for 3 hours in a secondary antibody, biotinylated anti-mouse Ig (1:100, Amersham). After another wash in PBS, sections were incubated for 3 hours in a tertiary antibody, streptavidin biotinylated horseradish peroxidase complex (1:100, Amersham), then washed in PBS and presoaked in 0.02% 3,3’-diaminobenzidine in 50 mmol/L Tris buffer (0.76% Trizma preset crystals), pH 7.4 (DAB solution). After 32 μL of 30% hydrogen peroxide was added to the DAB solution, the sections were incubated for another 7 minutes. This step was repeated, and the sections were then dehydrated, cleared, and coverslipped.

Thy-1 immunoreactivity was quantified with the use of commercial imaging software (MCID, Imaging Research) at ×200 magnification with the microscope and camera previously described. Approximately 15 to 20 measures were conducted on each section by determining the proportion of area that showed Thy-1 immunoreactivity. This was performed within a sampling area containing an approximately constant length of retina. Measures were averaged across sections for each eye. The staining for Thy-1 immunoreactivity was performed separately for the 3 postsurgical interval groups. To control for interbatch variability, the data for the 2VO eyes were expressed as a percentage of the mean score for the sham rats of the corresponding postsurgical interval.

The thicknesses of other retinal sublayers (outer nuclear layer [ONL], outer plexiform layer [OPL], inner nuclear layer [INL], inner plexiform layer [IPL]) were measured at ×400 magnification with the MCID software. The ONL contains the cell bodies of the photoreceptors, while the OPL contains their axonal/dendritic matrix. Hence, the thicknesses of the ONL and OPL reflect photoreceptor integrity. The density of the photoreceptors and their cell bodies was too high to permit stereological estimates. The INL contains the cell bodies of the bipolar, amacrine, and horizontal cells. The IPL is the synaptic matrix of these cells. Three or 4 hematoxylin-stained sections containing the central retina were selected for each eye. Multiple thickness measures (approximately 120 to 160 measures per section) were averaged for each layer on each section, and these were then averaged across sections for each eye.

One section containing a cross section of optic nerve was selected from each eye for Gallyas staining.\textsuperscript{18} Briefly, sections were deparaffinized and incubated overnight in 100% 1-propanol containing 0.8% distilled water and 1.2% concentrated sulfuric acid at 56°C. They were then rehydrated and reacted with 0.5% glacial acetic acid for 10 minutes and then with developer for 10 minutes. Finally, a 30-minute wash in 1% glacial acetic acid and then a 5-minute wash in propanol were followed by immersion in anise oil for 5 minutes before coverslipping.

All measurements were performed by an experimenter blind to the group membership of the retina.
Results

Of the 53 animals in the 2VO group, 18 (34%) eventually lost the direct and consensual pupillary reflex to light in both eyes. Another 13 animals (24%) eventually suffered unilateral loss of pupillary reflex, characterized by a lack of direct response in 1 eye (afflicted eye) accompanied by a lack of consensual response in the contralateral eye. In all cases of unilateral pupillary reflex loss, the afflicted eye retained the consensual response to light stimulation of the contralateral eye. In some cases, animals that eventually suffered bilateral loss of the pupillary reflex initially displayed unilateral loss of the pupillary reflex, followed by loss in the second eye. Loss of pupillary reflex often occurred immediately after surgery, most often within 3 days and in all cases within 7 days after surgery. The remaining 22 animals in the 2VO group, as well as all sham animals (n=20), displayed no pupillary reflex abnormalities.

The Table shows that the loss of pupillary reflex (unilateral or bilateral) was not determined by the light versus dark housing of the animal. The proportion of 2VO animals suffering from loss of normal pupillary reflex overall was 14 of 26 (54%) in the dark condition and 17 of 27 (63%) in the light condition. The χ² analysis indicated that the loss of reflex in 2VO animals occurred randomly across the light/dark groups (P=0.32).

For statistical analyses, the eyes could be classified according to group (sham versus 2VO), whether the eye showed the reflex (flex) or not (no flex), postsurgical interval (3, 15, or 90 days), and postsurgical housing condition (light versus dark). Since all of the sham-operated animals retained the reflex, there were 3 main groups (sham, 2VO/flex, and 2VO/no flex) that could be further subdivided. Analysis consisted of a 3×3×2 factorial design with group (sham, 2VO/flex, and 2VO/no flex), postsurgical interval (3, 15, and 90 days), and postsurgical lighting condition (light, dark) as the between-subjects variables. Since the eyes from the 3-, 15-, and 90-day light- and dark-housed sham groups showed no differences on any measure, their scores were collapsed into a single group (sham) for statistical comparisons. The data are shown in the top panel of Figure 1. Figure 4 (top row) shows representative hematoxylin-stained sections of the retina.

ANOVA of the RGCL cell numbers indicated no significant main effect of, or interactions involving, the light condition. There was a significant main effect of group (P<0.001). Scheffé’s test showed that overall, the 2VO/no flex group had fewer RGCL neurons (P<0.001) than sham and 2VO/flex groups, which did not differ. A priori ANOVA was conducted for the 2VO/flex, 2VO/no flex, and sham subgroups for the 3 different postsurgical intervals with the use of the Bonferroni correction for nonorthogonal comparisons when applicable. The 2VO/no flex eyes showed significantly fewer RGCL neurons than sham eyes by 15 days (P<0.05). By 90 days, an even more severe reduction was apparent, with a significant loss of cells compared with both sham (P<0.001) and 2VO/flex eyes (P<0.001).

The data for Thy-1 immunoreactivity shown in the bottom panel of Figure 1 consisted of scores for each eye expressed as a percentage of the corresponding sham mean for each postsurgical interval. Figure 4 (middle row) shows representative Thy-1 immunostained sections. Analyses of these data consisted of t test comparisons of the sham, 2VO/flex, and 2VO/no flex groups at each interval. The light/dark condition had no effect on Thy-1 immunoreactivity, and therefore the data were collapsed across this variable to simplify presentation. As shown in Figure 1, Thy-1 immunoreactivity was unaffected by 2VO at 3 days. However at 15 days the 2VO/no...
flex eyes showed significantly less immunoreactivity than both the sham (P<0.02) and 2VO/flex groups (P<0.001). The 2VO/flex and sham groups did not differ. At 90 days, Thy-1 immunoreactivity was undetectable in the 2VO/no flex rats, while again the sham and 2VO/flex groups did not differ.

Figure 2 shows the data for thickness of the ONL (top) and the OPL (bottom). ANOVA of the ONL thickness data indicated a significant effect of day (P<0.001), a significant group by day interaction (P=0.001), and a significant group by day by light interaction (P<0.001). The ONL thickness was unaffected until 90 days after surgery. Furthermore, post hoc (Scheffé’s test) comparisons revealed that only the 90-day 2VO/no flex eyes from the light condition showed thinning of the ONL. Notably, they had a thinner ONL than the sham eyes (P=0.004), the 90-day 2VO/flex eyes (P=0.03) from the light condition, and the 90-day 2VO/no flex eyes from the dark condition (P=0.027). The 2VO/no flex eyes from the dark condition were not different from the sham eyes. Hence, light exposure caused the reduction of ONL thickness in the 90-day 2VO/no flex eyes.

ANOVA of the OPL thickness data revealed a significant main effect of group (P<0.001), day (P<0.001), and a group by day interaction (P=0.002). As shown in Figure 2, OPL thickness was unaffected until 90 days after surgery. Scheffé’s test indicated that the OPL thickness of the 90-day 2VO/no flex group was significantly reduced compared with the sham (P<0.001) and 90-day 2VO/flex (P=0.001) groups. While this reduction was significant in the dark condition, it was more severe in the light condition.

The ANOVA of the INL thickness (data not shown) revealed only a significant main effect of day (P=0.02) and a day by light interaction (P=0.05). Scheffé’s test indicated that no group differed from sham eyes at any time point in any condition, and no 2 groups differed among all multiple comparisons.

ANOVA of the IPL thickness (data not shown) indicated a significant main effect of group (P<0.001) and day (P=0.006) as well as a significant group by day interaction (P=0.05). Scheffé’s test showed that the IPL of the 2VO/no flex eyes was thinner than that of both the sham eyes (P=0.02) and the 2VO/flex eyes (P=0.02) at the 90-day time point.

The optic nerve of the 2VO/no flex eyes was substantially shrunken compared with the 2VO/flex and sham eyes (Figure 4, bottom panel). Gallyas staining was not apparent in any of the sham ONs and was apparent, but very faintly, in only 2 of the 27 eyes in the 2VO/flex group. The remaining 24 eyes in the 2VO/no flex group showed substantial staining. As shown in Figure 3, Gallyas staining of the optic nerve of the 2VO/no flex animals was apparent by 3 days and at the 15- and 90-day time points as well. ANOVA revealed a significant group difference (P<0.001), with the 2VO/no flex group showing significantly increased staining over the sham (P<0.001) and 2VO/flex (P<0.001) groups. No significant effects of day or light were found.

Figure 4 shows representative hematoxylin-stained, Thy-1-immunostained, and Gallyas-stained sections from sham, 2VO/no flex dark-housed, and 2VO/no flex light-housed rats.

**Figure 2.** Top, Layer thickness measures of ONL of the retina. Bottom, Layer thickness measures of OPL of the retina. Symbols above bars designate groups that differed significantly. Abbreviations are as defined in Figure 1. Error bars represent SEM.

**Figure 3.** Percent area of optic nerve stained with Gallyas silver at 3, 15, and 90 days. *Significant difference from sham. #Significant difference from the flex group at the corresponding postsurgical interval. Abbreviations are as defined in Figure 1. Error bars represent SEM.
Discussion

Approximately 59% of the 2VO animals suffered permanent loss of the pupillary reflex in 1 or both eyes. When there was unilateral loss, the consensual response of this afflicted eye was spared, but the consensual response of the other eye was absent. Hence, the efferent pathway of the afflicted eye was functional, but its afferent pathway was dysfunctional before bifurcation.

Degeneration of the optic nerve was the only measure that temporally coincided with loss of pupillary reflex. Gallyas staining showed optic nerve degeneration by 3 days after surgery in all 2VO/no flex eyes. Conversely, 2VO/flex and sham eyes showed little or no staining. No differences in optic nerve staining were observed over the 3 postsurgical time points. At no time was there a difference between the optic nerves of the 2VO/no flex eyes from the light and dark conditions.

Cell number in the RGCL of 2VO/no flex eyes was significantly reduced after 15 days relative to the sham eyes. The reduction was more severe after 90 days. Thy-1 immunoactivity was also significantly reduced by 15 days and was undetectable by 90 days. Notably, the housing of the rats in light versus dark conditions did not affect these RGCL measures.

No significant differences between groups were noted for any of the retinal sublayers until the 90-day time point after surgery, when the 2VO/no flex eyes demonstrated thinning of the ONL, OPL, and IPL. However, thinning of the ONL occurred only in the light condition. Hence, loss of photoreceptor cells occurred much later than the loss of pupillary reflex and was mediated not by ischemia but apparently by light toxicity resulting from the inability of 2VO/no flex eyes to moderate the exposure to light.

In agreement with our results, others have also reported that the optic nerve suffers substantial degeneration after 2VO, as early as several days.\textsuperscript{11,19} Consistent with this finding, it has been noted that cerebral white matter is highly vulnerable to ischemia,\textsuperscript{20} and ischemic damage to optic nerve axons has been demonstrated with in vitro models.\textsuperscript{21,22} Also in agreement with our data, others have found that photoreceptor degeneration is not evident until several months after 2VO.\textsuperscript{9,12} However, these studies did not disclose the status of the pupillary reflex of their rats, and this could explain the reported variability in photoreceptor degeneration.\textsuperscript{12} To our knowledge, the temporal evolution of RGC damage after 2VO has not been previously recorded. However, a reduction in Thy-1 immunoreactivity thickness has been observed 9 months after 2VO, but again there was no information regarding the pupillary reflex of these animals.\textsuperscript{12} This is an important factor since, as was found here, Thy-1 immunoactivity RGCs were diminished only in rats that lost the reflex.

It has yet to be determined why only a subset of these 2VO rats loses the pupillary reflex and suffers visual system pathology. The structure of the circle of Willis varies considerably among rats.\textsuperscript{23} Strain differences in the effects of 2VO have previously been noted,\textsuperscript{24} although retinal pathology after 2VO has been reported in Wistar,\textsuperscript{24} Sprague-Dawley,\textsuperscript{13} and Long-Evans\textsuperscript{25} rats. It is possible that in some rats, larger-diameter vessels within the circle of Willis or larger posterior arteries would allow for reverse perfusion of the internal carotid and pterygopalatine arteries and thence the retinas. Other rats without this characteristic may suffer more severe ischemia and lose their pupillary reflex.

The 2VO model, with the strain of rat used in this study, closely parallels the ocular pathology of human carotid artery disease. Hence, it provides a useful model to investigate mechanisms of and therapy for the retinal degeneration associated with this disorder. As shown here, ischemic damage to the optic nerve and phototoxic death of the
photoreceptors are dissociable and reflect different pathological mechanisms. Optic nerve damage is an early event and is directly caused by ischemia. Recent work indicates that ischemia damages the optic nerve by reverse calcium influx via sodium channels.26,27 This can be prevented by the use-dependent sodium channel blocker mexiletine. If such channel blockers prevented optic nerve damage in the 2VO model, this would suggest their utility when there are ocular manifestations of human carotid disease. Photoreceptors could be protected by controlled light exposure or pharmacologically by compounds or drugs that attenuate phototoxicity, eg, antioxidants.28

It is important to note that retinal blood supply may also be sufficiently compromised in other rat ischemia models (eg, carotid occlusion plus hypotension, occlusion of vertebral and carotid arteries) to cause visual disturbance. This possibility is unexplored, although it has been reported that 15 minutes of global ischemia (4-vessel occlusion) causes degeneration of the optic tract and neuronal damage in the superior colliculus between 5 to 60 days after surgery.29 Visual dysfunction could result from transient global ischemia, thus accounting for the poor correlation between visuospatial behavior and measures of ischemic brain damage.30

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References

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