Experimental Glaucoma in Primates: Changes in Cytochrome Oxidase Blobs in V1 Cortex

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PURPOSE. To evaluate the effects of ganglion cell death from experimental glaucoma on the relative metabolic activities of neurons in the cytochrome oxidase blobs of V1 cortex in the macaque visual system.

METHODS. Monocular experimental glaucoma was induced in adult monkeys (Macaca mulatta and Macaca fascicularis) by laser application to the trabecular meshwork, increasing the intraocular pressure. After other experiments, the primary visual cortices were analyzed for functional excitation from surviving ganglion cells, as indicated by cytochrome oxidase histochemistry.

RESULTS. Cytochrome oxidase reactivity was uniformly reduced in blobs with input from the glaucomatous eye in a manner consistent with loss of known afferent inputs. The average size of glaucomatous blobs in layers 2 and 3 of V1 cortex was reduced by half.

CONCLUSIONS. Experimental glaucoma in monkeys reduces retinal input to the central nervous system, thereby reducing the metabolic drive to downstream targets, as indicated by the reduction in the size of cytochrome oxidase blobs in layers 2 and 3 of V1 cortex. The pattern of cytochrome oxidase loss within the blob was uniform, suggesting that all sources of afferent input to the blobs were affected by experimental glaucoma. (Invest Ophthalmol Vis Sci. 2001;42:358–364)

In a previous article we described the downstream reduction in metabolic activity in the lateral geniculate nuclei (LGN) and in the magnocellular (M) and parvocellular (P) input divisions of the layer 4C to V1 cortex after experimental glaucoma. It was concluded that as experimental glaucoma depleted the retina of ganglion cells, there was a corresponding metabolic reduction in both M-cell and P-cell pathways, with a significantly greater reduction in the P-cell rather than the M-cell pathway in layer 4C of V1 cortex. This article continues the presentation of the results of those experiments by describing the concurrent change in neuronal metabolism in the cytochrome oxidase (CO) blobs of the superficial layers 2 and 3 of the primate V1 cortex, the target of differential input from the two parallel pathways into layer 4C. The density and distribution of cytochrome oxidase reactivity (COR) is used as an indicator of the level of afferent visual signal from retinal ganglion cells to the blobs in layers 2 and 3.

CO is an essential mitochondrial membrane protein for brain oxidative metabolism, catalyzing the last step in the formation of adenosine triphosphate (ATP), the energy source for neuronal function. The brain tissue content of CO is inhomogeneously distributed and varies with the metabolic demand attendant with neuronal activation. In the afferent visual system of primates, histochemistry has shown CO reactivity to decrease monotonically with the duration of sensory visual deprivation by enucleation or by tetrodotoxin (TTX) blockade. Therefore, it is reasonable to expect CO reactivity to be at covariance with the level of activation of neurons in the monkey afferent visual system, including the blobs of superficial layers 2 and 3.

CO-rich blobs are oft described, and now well-known, metabolic features of the superficial layers 2 and 3 of primary visual cortex. In most primates, CO blobs are distributed in periodic rows directly above the center of the eye-dominance columns (ODC) of input layer 4C and are known to derive a major afferent input from the two laminar subdivisions, layer 4Ca and 4Cb, directly beneath. In turn, the input to these two subdivisions comes from the two major ganglion cell projection pathways, with the M-cell pathway (carrying primarily retinal information gathered by the large parasol retinal ganglion cells) projecting into layer 4Ca, and the P-cell pathway (carrying retinal information gathered by the small- to medium-sized retinal midget ganglion cells) projecting into layer 4Cb. In addition to these cortical afferents, the CO blobs get a direct thalamic input from the K-cell laminae of the lateral geniculate nucleus (carrying retinal information gathered by small bistratified ganglion cells). Although both layer 4C subdivisions send projections to superficial layers 2 and 3, the CO blobs are thought to receive a major input from layer 4Ca. Whereas the surrounding interblob areas get input from both divisions of layer 4C, the input from 4Cb contributes far more synaptic contacts. Because these studies have shown that there are multiple afferent inputs to CO blobs, and because we and Vickers et al. have shown that experimental glaucoma reduces CO staining in the macaque afferent visual system and we have shown a differential effect of experimental glaucoma on two of the primary afferent inputs to the blobs, we next measured changes in the size and metabolism of the CO blobs in these same monkeys.

MATERIALS AND METHODS

Subjects

Five adult monkeys (three Macaca mulatta and two Macaca fascicularis) were used in accordance with Office of Protection from Research Risks Public Health Service (OPRR-PHS) Policy on Humane Care and Use of Laboratory Animals (revised 1986), the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all research protocols had the approval of the institutional animal care and use committee (IACUC). The intraocular pressure (IOP) of the right eye of each subject was elevated by laser application to the trabecular meshwork using the procedures described by Quigley and Holman. Subsequently, IOPs were measured in the anesthetized subjects, typically
at weekly intervals for a period of approximately a year, using a handheld applanation tonometer. The animals had clear ocular media throughout. The details of the treatments, behavioral testing, electrophysiology, and metabolic changes in the lateral geniculate nucleus (LGN) and in layer 4C of V1 cortex have been referenced and presented earlier. All monkeys had severe experimental glaucoma-related visual damage as indicated by the Humphrey visual field map (HVFM; Humphrey Instruments, San Leandro, CA; see Reference 1, Tables 1 and 2, for background of three of the subjects: M. mulatta OHT9, OHT18, and OHT21).

Figure 1 is presented to indicate the level of glaucomatous damage in the five monkeys whose visual brains were analyzed in this article. The figure shows the HVFM for four monkeys. Figure 1A is a map (OHT19) typical of the threshold for detecting a visual defect, whereas the HVFMs of Figs 1B, 1C, and 1D are from monkeys used in the present study, and show severe defects from experimental glaucoma (OHT18, OHT9, and OHT21, respectively). Two other animals (M. fascicularis AL540 and AL203) were treated similarly, but systematic treatment and behavioral data were not collected on them. The visual field defects shown in Figures 1B, 1C, and 1D are indicative of advanced glaucomatous damage and a severe loss of retinal ganglion cells. Although counts of the loss in ganglion cells in these experimental animals are in preparation and incomplete, it is clear from our comparable counts in other animals treated similarly (e.g., see Reference 19 Fig. 2) that all animals analyzed in this report had advanced glaucomatous retinal damage.

Measurements of CO blobs were made in six cortical slices, four ipsilateral and two contralateral to the glaucomatous experimental eye. The measurements were made at a cortical locus corresponding to the upper temporal retinal representation of the hemifield, approximately 7° to 10° from the fovea; a retinal representation generally showing severe but not total loss of visual sensitivity.

Tissue Preparation

At the end of the behavioral or electrophysiological study, the animals were killed by overdose (100 mg/kg) of pentobarbital sodium (Nembutal; Abbott, Abbott Park, IL), and exsanguinated with 2 l of saline followed by 2 l of a 2% paraformaldehyde–0.5% glutaraldehyde fixative in phosphate buffer (pH 7.4). The brain and optic nerves were removed and (in most cases) the visual cortices were dissected and gently flattened on a glass slide. The tissue was refrigerated overnight in the fixative before beginning a sucrose dehydration gradient of 10%, 20%, and 30%. The visual cortices were embedded in aluminum foil cups filled with TissueTek (Miles, Elkhart, IN) and frozen by lowering into an acetone bath cooled by liquid nitrogen. Tangential sections of 30-μm thickness were collected and stained for the histochemical localization of CO according to the protocol of Wong–Riley. The CO-stained sections were mounted on gelatinized slides, dehydrated, and coverslipped (Permount; Fisher Scientific, Fairlawn, NJ).

Optical Measurements

The CO-stained sections were homogeneously back illuminated, and a digital image was captured at a 1200-pixel resolution with a camera (DCM1; Polaroid, Cambridge, MA), which was linear with optical density, with an R = 0.99. The density of the COR was scaled 0 to 255, where 0 = opacity and 255 = the incident light. Measurements were taken before any filtering or contrast enhancement. COR = I − T, where I was the incident light (nominally, a value of 255), and T was the light transmitted through the tissue containing the CO-reaction product. The value of COR from a blob connected with the glaucomatous experimental eye (CORg) was always compared in the same section with the value of COR measured in the directly adjacent blob connected with the normal companion eye (CORn). The ratio, CORg/CORN, constituted the primary data from which mean values and variances were calculated. The mean COR and SD of a closed contour, drawn by eye to best outline the blob, were recorded for each blob and compared with the values obtained in the companion blob with pri-
mary input from the opposite eye. Thus, the ratio of \( \text{COR}_G/\text{COR}_N \) was formed. A minimum of 10 such ratios was then averaged for each tissue location. Most often, the ratio \( \text{COR}_G/\text{COR}_N \) was expressed as a percentage reduction of COR, relative to that in the companion site that had input from the normal eye.

For the relative size measurement, the numbers of pixels contained within the closed contour of the tracing of the CO blob image was compared. It is recognized that there is no generally accepted objective criterion for drawing the CO blob boundaries, because there is a characteristic decreasing COR gradient from the blob center toward the edge.\(^{14}\) Therefore, we present a statistical comparison of the subjective sizes of pairs of CO blobs, collected by a technician naive to the purposes of the study, and the results evaluated by the paired \( t \)-test.

The brain section image was processed using the University of Texas at San Antonio Image Tool 2.0 image analysis software. With the reticulated CO pattern characteristic of layer 4A as a reference, the perimeters of the CO blobs in layer 3 were traced by eye and the numbers of pixels within the enclosed perimeter taken as an index of the relative area of blobs with input from the normal and the glaucomatous eye. The average area and relative COR were measured for pairs of CO blobs, one from the normal left (ODC), paired with the adjacent CO blob from the ODC of the glaucomatous right eye. A minimum of 10 pairs was measured from the V1 cortices of each of five of the experimental monkeys. The primary data were imported into a spreadsheet (QuattroPro; Corel, Ottawa, Canada) for computation and graphing, whereas statistical comparisons were made using the paired \( t \)-test (SigmaStat; Jandel Scientific, San Rafael, CA).

To describe the distribution of relative COR within the blob, pairs of normal and companion glaucomatous blobs were scanned along a line orthogonal to the course of the eye dominance columns. Smoothing the resulting noisy curves was achieved by subjecting the data to a 3-point rolling average to generate a 48-point profile for 10 pairs of blobs.

To present a different and enhanced view of the density, size, and distribution of COR within the blobs, the following operations were performed on selected blob fields. First, the density range of the image was inverted so that the low-intensity blob COR density signal, represented by the dark COR blobs, became high-intensity values. Next, the pixel values in the holes representing the blood vessels were replaced by the average value of the immediately surrounding pixels. A surface plot was then made so that the pixel intensity was coded both by height of the surface and by color. An azimuth and elevation was chosen to optimize inspection of differences between rows of blobs. In practice, low-pass filtering was often performed to accentuate the major features over high-frequency variations.

To fill and replace the blood vessel holes, two different protocols were used with approximately equal success. In the first protocol, the holes were selected in the image management software (Photoshop; Adobe, San Jose, CA) by selecting that range of intensities. A mask was created corresponding to the holes. The remainder of the image without the holes was then blurred by several passes of a median filter. The calculation facility of the software program was then used to replace the masked area corresponding to the holes with corresponding portions of the blurred image. This replaced the white holes with a local average of the surrounding area. In the second protocol, a custom "zippering" routine was implemented in a statistical analysis software program (The MatLab; MathWorks, Natick, MA). By this means, an upper threshold was imposed that distinguished the high-intensity holes from the darker tissue. The routine then replaced pixels exceeding this threshold with the value of their nearest neighbor not exceeding the threshold. By this mechanism, the surrounding regions ‘flooded’ the holes with their values. This procedure was more robust in dealing with a wide variety of tissues but sometimes replaced the holes with values somewhat lighter than the near background, probably because of light scattering near the holes.

**RESULTS**

The effects of experimental glaucoma on the neural metabolism within the CO blobs of V1 layers 2 and 3 are illustrated in the two panels of Figure 2. The top panel is a view of a tangential section showing a view of the rows of CO blobs that lie directly above a comparable field of ODC stripes in layer 4C (lower panel). Parallel rows of encircled CO blobs, running top to bottom, are illustrative of the tracings made by eye for the estimation of the relative area of the CO blobs having input from the normal eye (large arrows, N) and from the glaucomatous eye (small arrow, G) indicated in the lower panel. The CO blobs associated with input from the normal left eye are characterized by a band of COR of a higher density than background. The adjacent row of CO blobs is associated with afferent input from the glaucomatous right eye and is characterized by smaller CO blobs without the interconnecting band of extrablob COR. In the lower panel, the CO-rich ocular dominance columns from layer 4C, having input from the normal eye (N), and the CO-poor columns with afferent input from the glaucomatous right eye (G), are seen juxtaposed to comparable rows of blobs in the upper panel.

Figure 3 shows the relative COR values from the six visual cortices of the individual experimental animals for blobs associated with input from the glaucomatous eye (G) with those blobs with input from the normal eye (N). The average relative reduction in COR in blobs associated with the glaucomatous right eye for all the monkeys was 9% (range, 2–14%). The average COR tended to be reduced in the blob associated with the glaucomatous eye, with four of the six comparisons being significant reductions (paired \( t \)-test; \( P < 0.01 \)).

Normally, the level of COR in the blobs is approximately the same in both visual cortices, as shown in the normal eye data.
from OHT18 (R, L) displayed in Figure 3. The overall percentage of reduction in COR was insignificantly greater between blobs on the ipsilateral right cortex (14%) compared with those blobs in the cortex contralateral to the glaucomatous eye (12%), as might be expected owing to the characteristic spread of the glaucomatous lesion.1

Overall, the average COR data for the five experimental monkeys (Fig. 3) shows that there was a significant reduction in the COR between the blobs having input from the glaucomatous right eye (RE) compared with those blobs having input from the normal left eye (LE). The distribution of the loss in COR within the blob is illustrated in Figure 4A, showing the COR profiles of 10 blob pairs. The shape of curve of the means and SD for 10 glaucomatous blobs was comparable with that of the companion 10 normal blobs, differing only in the lower relative level of COR in the glaucomatous blobs throughout. This suggests a uniform loss in COR throughout the blob, which is borne out in Figure 4B, showing a linear regression fit to the 24 mean values from the center of the blob into the interblob space. The parallel COR data and the regression curves attest to a uniform loss in COR throughout the glaucomatous blob.

Experimental glaucoma dramatically reduced the relative size of the CO blob, as is shown in Figure 5. In every case, the size of the CO blob receiving input from the glaucomatous eye was significantly smaller than the normal companion CO blob (six paired t-tests: t > 9.0, df = 22, P < 0.001) with an average shrinkage of 53%. The smallest amount of shrinkage of the CO blob is seen in the left visual cortex (L) of OHT18, where the average reduced size was still a significant 23%. Therefore, experimental glaucoma dramatically reduces the spatial domain of the affected eye in superficial primary visual cortex, with a specific impact on the metabolic activity in the CO blobs.

The reduction in blob size and COR distribution is apparent in the rendering of the tangential surface plot of a field of CO blobs shown in Figure 6. Figure 6A shows the unfiltered image of a field of CO blobs from layers 2 and 3, with the ODCs running almost vertically. The dashed line contour connects four blood vessels that serve as fiducial marks for spatial registration of the Figures 6A through 6D. Figure 6B shows the contrast-enhanced field of CO blobs for better viewing. Figure 6C shows the density reversal of the image, with the holes of the blood vessels appearing as black spots and the COR appearing white. Figure 6D shows the image after the thresholding and application of a hole zippering routine, which effectively removed the blood vessel and hole artifacts from the image, leaving the COR displayed as a light-gray–scale image. The rows of CO blobs associated with the experimental glaucoma of the right eye (R) and those associated with the normal eye (L) are shown.

**Figure 5.** Relative percentage reduction in blob size of five monkeys with experimental glaucoma. The cortex ipsilateral (L) to the glaucomatous eye (R) had reductions in excess of 50%, reflecting the nature of the spread of the scotoma.
eye (L) are marked, as is the subfield of CO blobs presented in Figure 6E.

Figure 6E is a surface plot of the subfield of CO blobs marked in Figure 6D. The COR density is color-coded, and the field of blobs appears as rows of parallel peaks. Alternate rows of blobs associated with the normal eye appear as robust, broad peaks interconnected along the row by a higher level of COR, whereas the adjacent rows of blobs connected with the glaucomatous eye appear much smaller and do not have the interconnecting bridge of COR.

In summary, as the afferent input from ganglion cells to the visual brain is reduced by experimental glaucoma, the CO blobs of the superficial layers of primary visual cortex become smaller in a manner consistent with a uniform loss of metabolism within the blob.

**DISCUSSION**

These results show that experimental glaucoma dramatically altered metabolic activity as well as the spatial domain of the affected eye in superficial layers of primary visual cortex. As retinal ganglion cells became nonfunctional (and eventually died), the entire downstream chain of neurons became metabolically reduced, and the cortical spatial domain formerly controlled by the affected eye became quiescent. That is not to say that these neurons were inactive, just that they had lost much of their primary afferent signal and were relatively reduced in their neural activity, as the CO marker for neuronal metabolism indicated.

The effects of glaucoma on the superficial CO-rich blobs of the V1 cortex are interesting in that the pattern of loss in COR
suggests a uniform loss throughout the blob. Trusk et al.\textsuperscript{14} and Edwards et al.\textsuperscript{20} have characterized the density and pattern of change in COR within the normal CO blob, showing that COR is highest in the blob center, indicating that there is normally an underlying anisotropy in metabolic activity within the blob. Trusk et al.\textsuperscript{14} also showed that the COR reduced uniformly throughout the blob after deafferentation by enucleation, TTX blockade, and monocular eyelid suture conditions similar to that associated with deafferentation by glaucoma as we have shown here. Therefore, a uniform reduction in COR throughout the blob accounts for both the smaller blob size and the consistent COR profile in the blobs associated with the glaucomatous eye reported here.

Because the CO blob gets multiple inputs, it is surprising that the effect of glaucoma was uniform throughout the blob. Because we have shown\textsuperscript{1} that both P- and M-cell LGN layers supplying signals to V1 are metabolically\textsuperscript{,}but differently, inactivated by glaucomatous blockade, it is worthwhile to consider the contribution of those other pathways having input to the CO blobs.

One obvious source of stimulation to the CO blobs of layers 2 and 3 is the direct thalamic input from the koniocellular (K)-cells from the extralaminar zones of the LGN.\textsuperscript{21–23} As the P- and M-cell input to the blobs weakens and the blob shrinks, the K-cell input could sustain elevated metabolism within the shrinking blob. However, because the K-cell itself gets a major afferent input from a third morphologic and functional class of retinal ganglion cell, the small bistriatified ganglion cell\textsuperscript{25,24} (thought to carry blue-yellow color information; see recent review\textsuperscript{25}), it too would probably be subject to the same deleterious effects of glaucoma. Although the K-cell receives other input (first from the superficial layers of the superior colliculus [SC], as well as from the parabigeminal nucleus [PG]), it is hard to see how these indirect afferent routes into the CO blobs could sustain metabolic activity, because both these sources are themselves dependent on retinal ganglion cell input. For example, the SC superficial layers get a large projection from V1, layer 5, which would be downstream of the impaired P-, M-, and K-cell geniculocortical projection just discussed. Moreover, it would be expected that advanced glaucoma would weaken the direct ganglion cell input to SC in a similar manner to the effects shown for the geniculocortical projection.

Moreover, if one uses the cell size argument in the sequence of impairment and death of ganglion cells in glaucoma\textsuperscript{26} (i.e., that it is the ganglion cells with the largest soma size that are first affected in glaucoma) the small bistriatified ganglion cell supplying input to the K-cell would have no particular survival advantage, in that these ganglion cells have some sizes intermediate to midget and parasol ganglion cells having input to the P- and M-cell LGN layers, respectively. Therefore, it is unlikely that K-cells would have any survival advantage in glaucoma.

Because it has been suggested that the collective input to the blobs from the three LGN sources (layers 4Ca, 4CB, and K-cell) represents less than 20% of the synaptic connections within the CO blob,\textsuperscript{25} most of the synaptic contacts within the blob must come from intracortical sources. For example, if the mosaic of blobs has some binocular interconnections (either excitatory or inhibitory) the active blobs connected with the normal eye could affect stimulation to the CO blob connected with the glaucomatous eye. What evidence is there for such a pathway?

Several studies have shown that there are extensive intracortical lateral projections (spreading axons with periodic terminations) within the superficial layers of V1 of primates. Orthograde tracers have been injected within the layer 3 CO blob, and the characteristics of the lateral axonal spread described.\textsuperscript{27} Malach et al.\textsuperscript{28} showed that biocytin injections within one CO blob marked axonal spread that tended to contact adjacent blobs connected with the same eye, but not to the blobs connected with the opposite eye. However, using similar methods, Yoshioka et al.\textsuperscript{29} have shown that these lateral projections spread predominantly orthogonal to the course of the ODCs, where axonal branches contact most of the adjacent CO blobs. These two studies reinforce the original paper on the subject by Livingstone and Hubel\textsuperscript{30} who used horseradish peroxidase to show that blobs are connected with blobs, and nonblob areas are interconnected with other nonblob areas. These studies agree that there is a statistical preference of these lateral projections to link CO blobs in ODCs associated with the same eye, but that there are significant exceptions observed in each study. Therefore, although most of the lateral interconnections seem to be between like compartments (e.g., blobs to blobs; interblobs to interblobs, and interblobs within ODCs of the same eye) a significant percentage of the axonal terminals make contact with the CO blobs associated with the opposite eye. Such reciprocal contacts between pairs of CO blobs could support interblob neural coherence and provide a synchrony between ODC domains analyzing the same part of visual space. It could be speculated further that a neural coherence is necessary to knit together a higher order binocular perceptual map, with the CO blobs serving as neuronal fiducial points for keeping the monocular maps synchronized and functionally aligned. From our results, there seems to be no differential spatial input from these sources, in that the loss in COR appears to uniform throughout the blob. As the afferent inputs to the blob from layers 4Ca and 4CB and from the K-cells of the LGN fail with the progressive death of the retinal ganglion cells, the lateral input from the adjacent column driven by the normal eye are apparently insufficient to sustains metabolic demand within CO blobs of the glaucomatous eye. Of course, a strong counter argument to this possibility is the absence of binocularity of neurons within the blobs.

Experimental glaucoma produces a differential reduction in the layer 4C input sublaminae, with a greater loss in 4CB than in 4Ca.\textsuperscript{1} This result, which we reported earlier, was consistent with what we had found in the LGN (that experimental glaucoma had a greater effect on the metabolism of the P-cellular pathway, than on the M-cell pathway), but incompatible with the earlier suggestions of Glovinsky et al.\textsuperscript{26} who presented evidence to the contrary—that the larger parasol ganglion cells, which make up the primate M-cell retinogeniculocortical pathway are affected first and more severely in glaucoma in humans and in experimental glaucoma in monkeys. The results occurring in the CO blobs reported herein suggest a uniform reduction in COR throughout the blob, consistent with a uniform spatial projection to the blob from the 4Ca and the 4CB sublaminae. By contrast, Edwards et al.\textsuperscript{21} have reported recording from a small number of neurons within the center of the blob having a higher contrast sensitivity (a characteristic of the M-cell pathway) than those neurons near the periphery of the blob, suggesting an anisotropy in the inputs from the M-cell and P-cell pathways. The uniform reduction of COR throughout the blob reported here provides no evidence for a differential spatial projection of M- and P-cell input within the blob and is consistent with the results of Trusk et al.\textsuperscript{14}

These results further extend the description of the pathophysiology of glaucoma along the afferent chain of anatomic sites from the eye to the superficial layers of primary visual cortex.

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References


