Visual Impairments Following Term Neonatal Encephalopathy: Do Retinal Impairments Also Play a Role?

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METHODS. A rat model of term neonatal hypoxic-ischemic (HI) encephalopathy (Vannucci model) was used. Hypoxia-ischemia was induced by a left common carotid ligation followed by a 2-hour period of hypoxia (8% oxygen) in Long-Evans rat pups at postnatal day 10 (P10). Sham operated rats served as controls. Retinal function was assessed at P30 and P60 by electroretinograms (ERGs), after which retinal histology was performed. Retinocortical function was assessed with visual evoked potentials (VEPs) at P60 and subsequently brain histology was performed.

RESULTS. The ERGs of the HI animals at P30 and P60 demonstrated a significant reduction in the scotopic and photopic b-wave amplitudes, but a preserved a-wave amplitude. The retinal histology of the HI animals confirmed that the photoreceptor layer remained intact, whereas the inner layers of the retina were damaged. The HI animals also showed reduced VEP P100 amplitudes, which correlated with reduced left cerebral hemisphere surfaces. There was no correlation between the severities of retinal versus cerebral injuries.

CONCLUSIONS. Our findings suggest that term neonatal encephalopathy resulting from HI induces functional and structural damages to the inner retina, while relatively sparing the photoreceptors. These findings raise the possibility that retinal injuries may contribute to visual impairments with or without the presence of brain injury in term asphyxiated newborns and, thus, warrant further studies with humans and animals to better understand the disease process.

Keywords: birth asphyxia, brain, neonatal encephalopathy, retina

Despite improvements in neonatal care, birth asphyxia remains a serious condition that causes significant mortality and long-term morbidity, such as cerebral palsy, mental retardation, and visual impairments (including blindness). Although birth asphyxia and the resulting neonatal encephalopathy in term newborns have been extensively studied, relatively few studies have explored their impact on the retina and visual function. However, loss of vision following birth asphyxia is one of the most common causes of pediatric visual impairments in developed countries. These visual impairments have been largely attributed to injuries along the intracerebral visual pathways (i.e., the optic nerves, optic radiations, primary visual cortex, visual associative cortical areas, and/or visual attention pathways) rather than injuries to the retina, although in some studies abnormal ocular findings have been reported. One study reported transient subnormal electroretinogram findings within 1 year following birth asphyxia, while other studies found normal electroretinograms (ERGs) with abnormal visual evoked potentials in asphyxiated newborns. All of the above make it difficult to appreciate if the retina is at risk of direct hypoxic-ischemic (HI) injury in asphyxiated newborns.

The retina is the organ that has the highest oxygen consumption per volume in the body. In addition, much like the brain, the developing retina is highly sensitive to variation in oxygen levels, as demonstrated by the abundant literature on the retinopathy of prematurity, including the animal model (i.e., oxygen-induced retinopathy) of this disease, as well as by several studies using different premature animal models of HI, which have reported functional and/or structural damages to the retina.

The Vannucci model (unilateral common carotid ligation followed by hypoxia), which was originally developed to study brain injury in the context of neonatal HI encephalopathy (HIE), also must be a valid model to simultaneously study the effect of HI on the retina, since the occlusion of the common carotid artery also disturbs the blood supply to the retina. Thus, we hypothesized that in addition to the brain injury, HI in this animal model also will induce injury to the retina. Furthermore, we hypothesized that the retinal injury will be most pronounced in the inner retina, based on the findings obtained in other hypoxic or ischemic retinopathy models, such as the oxygen-induced retinopathy. The present study was designed to investigate the detailed effects of neonatal HI on retinal function and structure, and compare them to the retinocortical visual function and brain structure using a rat model of term neonatal HIE.
MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the local animal care committee. Adult female Long-Evans rats with their male-only litters (Harlan Laboratories, Frederick, MD, USA) were received in the animal facility, housed under standard environment, and allowed food and water ad libitum. Rat pups remained with their mother until weaning at postnatal day 21 (P21).

Induction of Term Neonatal HIE

A well-established rat model of term neonatal HIE (Vannucci model), combining a unilateral common carotid artery ligation and a 2-hour exposure to hypoxia in 10-day-old rat pups, was used for the experiments, since this model is recognized to mimic the brain injuries observed in human term asphyxiated newborns. The common carotid artery gives rise to the ophthalmic artery, which feeds the two major blood vessels supplying the retina, that is, the choroidal and retinal arteries. For the ligation, 10-day-old male Long-Evans rat pups were weighed and then deeply anesthetized with an intraperitoneal injection of fentanyl (0.2 mg/kg) and midazolam (1 mg/kg) to the point of unresponsiveness to noxious stimulation. Anesthesia was reversed with an intraperitoneal injection of flumazenil (1 mg/kg). Pups were allowed to recover for approximately 1.5 hours after surgery, and then were placed in a sealed hypoxia chamber (Plastic Concepts, North Billerica, MA, USA). The chamber was gradually filled with nitrogen until a level of 8% oxygen was reached, which was maintained for 2 hours. Pups then were allowed to recover for 30 minutes and returned to their mother. Rats undergoing the whole procedure were considered as the HI group (n = 14). Sham operated rats (identical procedure as the HI group, but not the ligation and the hypoxia) served as the control group (n = 10). Rats undergoing the carotid ligation but not the hypoxia served as the ischaemia-only group (n = 4), while the right eyes of HI rats served as the hypoxia-only group.

Retinal Function

Full-field flash ERGs were recorded at P30 (acute effect) from the left (i.e., ipsilateral to the carotid ligation) and right (i.e., contralateral to the carotid ligation) eyes of HI animals, and from the left eyes of the control and ischaemic-only animals, using a data acquisition system (AcqKnowledge; Biopac Systems, Inc., Goleta, CA, USA) as previously described. Follow-up ERGs were recorded at P60 (chronic effect). Briefly, following a 12-hour period of dark-adaptation, the rats were weighed and then anesthetized with an intraperitoneal injection of ketamine (85 mg/kg) and xylazine (5 mg/kg). The pupils were dilated using 1 to 2 drops of tropicamide (Mydriacyl; Alcon Canada, Mississauga, ON, Canada), and the cornea was anesthetized with 1 to 2 drops of 1% of propracaine hydrochloride (Alcaine; Alcon Canada, Inc.). The rats then were positioned on their sides inside a recording chamber. All procedures were done under a dim red light. Scotopic ERGs were evoked to flashes of increasing intensities ranging from -6.3 to 0.9 log cd·s·m⁻² (0.3 log unit increment; interstimulus interval, 10 seconds; averages, 3–5 flashes; bandwidth, 1–1000 Hz), while photopic ERGs were recorded after 20 minutes of light adaptation (background, 30 cd·m⁻²; stimulus intensity, 0.9 log cd·s·m⁻²; interstimulus interval, 1 second; averages, 20 flashes).

The maximum mixed rod-cone a-wave (measured from the prestimulus baseline to the trough of the a-wave) and b-wave (the trough of the a-wave to peak of the b-wave) amplitudes and the photopic b-wave amplitude (baseline to b-wave peak) were measured using the AcqKnowledge software (Biopac Systems, Inc.). In cases where the peak of the b-wave could not be determined, the amplitude of the b-wave was considered as that measured at the time when the b-wave peaks in control animals. The maximal rod-mediated b-wave amplitude (referred to as rod Vmax) was estimated using a sigmoidal fit of the luminance-response function curve as reported previously.

To date, the ERG is the only objective mean to noninvasively assess retinal function. It is recognized that the ERG a-wave is generated by the photoreceptors (outer retina), while the b-wave represents electrical activities originating from the inner retina (most probably interactions between bipolar cells and Müller cells). Previous studies on ischemic retinopathy models, such as bilateral common carotid ligation or 4-vessel occlusion models, revealed an impaired inner retinal function (i.e., depressed b-wave) with relative sparing of the outer retinal function (i.e., the a-wave). Previous studies using the Vannucci model showed variability in the severity of brain injury. Consequently, given that variability in retinal injury could potentially occur, we postulated that a complete destruction of inner retinal function would abolish the ERG b-wave and, thus, yield a b-wave amplitude to a-wave amplitude ratio (b/a-wave ratio) smaller than 1 (severe phenotype), while a b/a-wave ratio between 1 and 2 would suggest a mild phenotype given that the normal b/a-wave ratio is approximately 2. Following the same logic, ratios of approximately 1 would identify an intermediate phenotype.

Retinocortical Function

After photopic ERG recordings (20 minutes of light-adaptation), visual evoked potentials (VEPs) were recorded with a subdermal needle electrode placed on the scalp over the occipital cortex (where the midline and interaural line cross; background, 30 cd·m⁻²; stimulus intensity, 0.9 log cd·s·m⁻²; interstimulus interval, 1 second; averages, 100 flashes). In control animals, only the left eye was stimulated (since equal VEP responses were assumed for the left and right eye stimulation). In HI animals, monocular VEP responses were obtained to compare the left and right retinocortical pathways. The P100 wave was identified as the most prominent positive peak occurring at a latency of approximately 100 msec, and its amplitude was measured from the trough immediately preceding the P100 to the peak of the P100.

Retinal Structure

After the ERG and VEP recordings at P60, the animals were euthanized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde. The left eyes of the HI and control animals were enucleated and immediately immersed in 4% paraformaldehyde for 3 hours, before the removal of the cornea and lens. The eyecups then were reimmersed in 4% paraformaldehyde and left overnight at 4°C on an orbital shaker. On the following day, the eyecups were washed 3×5 minutes in 0.1 M phosphate buffer. The eyecups then were incubated in a solution of 1% osmium tetroxide for 3 hours, followed by 3×5-minute washes in 0.1 M phosphate buffer, and sequential immersions in 50, 80, 90, 95, and 100% ethanol, and propylene oxide before embedding (Epon resin; Mecalab, Montreal, QC, Canada). The embedded
eyes were sectioned into 1-μm thick sections along the superior-inferior axis at the level of the optic nerve head, collected on glass slides, and stained with 0.1% toluidine blue. Images were taken with a microscope equipped with a digital camera (Carl Zeiss Microscopy GmbH, Jena, Germany) combined with a ×40 objective. The thicknesses of the total retina and of each retinal layer were measured at approximately 1000 μm from the optic nerve head in the inferior retina using AxiosVision software (Version 4.8.2.0; Carl Zeiss Microscopy GmbH). For retinal reconstruction, retinal segments of 75 μm in width, taken at every 340 μm along the entire length of the superior and inferior retinas, were assembled side by side (Adobe Photoshop; Adobe Systems, Inc., San Jose, CA, USA) to yield a panretinal view. Then, the thickness was plotted against eccentricity to obtain the spider graphs, centered with the optic nerve head; the superior retina represented on the left of the optic nerve head and the inferior retina represented on the right of the optic nerve head.

Brain Structure

The brains were extracted after the perfusion described above and postfixed in 4% paraformaldehyde solution overnight at 4°C, and then they were cryoprotected in 30% sucrose and serially sectioned into 16-μm coronal sections. Anterior sections were collected at −0.36 mm from Bregma (anterior commissure area) and posterior sections at −2.16 mm from Bregma (hippocampus area). After hematoxylin and eosin staining using standard protocol, brain morphology of posterior sections was examined with a light microscope (Leica DM4000B LED; Leica Microsystems, Wetzlar, Hessen, Germany) with a ×5 objective. For each section, overlapping microphotographs were captured using a digital camera attached to the microscope (Leica DFC450C, Leica Microsystems). These pictures were then stitched together using a panoramic image stitching software (Microsoft Research Image Composite Editor) to yield a panretinal view. Then, the thickness was plotted against eccentricity to obtain the spider graphs, centered with the optic nerve head; the superior retina represented on the left of the optic nerve head and the inferior retina represented on the right of the optic nerve head.

Data Analysis

One-way ANOVA followed by Dunnett’s post hoc comparison tests (for significant ANOVA results) were performed to assess the differences between the control and experimental groups. Regression analyses were performed to assess the correlation between the left/right hemisphere surface area ratio (brain structure) and P100 amplitude (brain function), P100 and photopic b-wave amplitudes (retinal function), and the left/right hemisphere surface ratio (brain structure) and the total retinal thickness measured at 1000 μm from the optic nerve head (retinal structure). A P value < 0.05 was considered as statistically significant. All statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Effect of Neonatal HI on Retinal Function

The rat pups exposed to HI at P10 presented with variable degrees of changes in their retinal function at P30 and P60. As shown in Figure 1A, compared to control animals, the mixed rod-cone ERGs of the ipsilateral (left) eye of the HI animals showed three distinct morphologies, namely: (1) the mild HI group with a b-wave-to-a-wave ratio (2.16 ± 0.28, n = 5) similar to the control (2.51 ± 0.07), i.e., b-wave being greater than the a-wave), (2) the moderate HI group with a b-wave-to-a-wave ratio close to 1 (i.e., b-wave being approximately equal to the a-wave; 1.04 ± 0.16, n = 5), and (3) the severe HI group with a b-wave-to-a-wave ratio less than 1 (i.e., b-wave being smaller than the a-wave; 0.44 ± 0.12, n = 6). All subsequent comparisons were made according to these subgroups. As illustrated in Figure 1A, the amplitudes of the rod mediated b-wave (rod V_{max}) and the photopic b-wave were either similar to those of the control animals (in the mild HI group) or reduced to varying extents, depending on the subgroup (in the moderate and severe HI groups), a finding that is best summarized with the data presented in Figure 1B or Table 1.

Effect of Neonatal HI on Retinal Structure

The HI-induced changes in the retinal function were accompanied by changes in the retinal structure in the left eyes of the HI animals (Fig. 3A). The total retinal thickness of the left eyes was significantly different between the groups (Fig. 3B). The moderate and severe HI groups showed a significant decrease in the total retinal thickness (P < 0.001) compared to the control. The mild HI group did not show a significant difference.

Analysis of the thicknesses of individual retinal layers also revealed significant differences between the groups. The inner retinal layers and the outer plexiform layer (OPL) were damaged to varying extents in the moderate and severe HI groups. That is, the moderate HI group showed a thinning of these layers, whereas the severe HI group showed a near complete destruction of these layers with only one band of the inner nuclear layer (INL) cells remaining below the OPL (Fig. 3A). Aforementioned observations were supported by the data shown at Figure 3B and Table 2. The moderate and severe HI groups showed a significant decrease in the thickness of the inner retinal layers (i.e., INL, P < 0.001; inner plexiform layer [IPL], P < 0.001; retinal ganglion cell/laser layer [RGC/FL], P < 0.01). These groups also showed a significant decrease in the thickness of the OPL (P < 0.001). The mild HI group showed no significant
differences in the thickness of the inner retinal layers and the OPL compared to the control. In contrast, the thickness of the photoreceptor layers (i.e., the outer segment [OS], inner segment [IS], and outer nuclear layer [ONL]) did not change in the mild and moderate HI groups, but it increased significantly in the severe HI group \((P < 0.05)\) compared to the control. Finally, the thickness of the RPE did not differ significantly between the groups \((P = 0.44)\).

In addition, the distribution of inner retinal (or postphotoreceptor) injury was heterogeneous along the superior-inferior axis of the retina, the damage being more pronounced at the center and the far periphery compared to the mid-periphery.

**FIGURE 1.** Neonatal HI–induced functional impairments of the ipsilateral (left) retina at postnatal day 30 (P30). (A) Representative scotopic (mixed rod-cone and rod \(V_{\text{max}}\)) and photopic ERGs. Horizontal and vertical calibrations in milliseconds (ms) and \(\mu\text{Volts (}\mu\text{V)}\), respectively. \(\text{Vertical arrows correspond to stimulus onset. The a-wave and b-wave are indicated by}\ a \text{and} b, \text{respectively. The ERGs from the left eyes (i.e., ipsilateral to the carotid ligation, OS; solid line) of the HI animals presented three phenotypes: mild (mixed rod-cone b-wave > a-wave), moderate (mixed rod-cone b-wave > a-wave), or severe (mixed rod-cone b-wave < a-wave). The ERGs from the right eyes (i.e., contralateral to the carotid ligation, OD; dotted line) of the HI animals and the left eyes of the ischemic animals appeared similar to those from the left eyes of the control animals. (B) Amplitude measurements of the ERG waves. Clear bars, left eyes (OS); shaded bars, right eyes (OD). Mean ± SD. The HI subgroups and ischemic group compared to the control group: \(* P < 0.05\). Only the right eyes of the severe HI group showed a significant difference in the a-wave amplitude compared to the control. The amplitudes of the mixed rod-cone b-wave and rod \(V_{\text{max}}\) were significantly decreased in the left eyes of the moderate and severe HI groups, and increased in the right eyes of the severe HI group, but not different in the right eyes of the mild and moderate HI groups, and in the left eyes of the ischemic group. The amplitude of the photopic b-wave was significantly decreased in the left eyes of the moderate and severe HI groups, but not in the other groups.**
### Effect of Neonatal HI on the Brain

The HI animals showed a variable degree of injury on the left (ipsilateral to the carotid ligation) hemisphere (Fig. 5). The left-to-right hemisphere surface ratio was positively correlated with the P100 amplitude driven by the right eye ($r^2 = 0.48$, $P < 0.01$; Fig. 6A). There was no correlation between the P100 amplitude driven by the right eye and the b-wave amplitude of the left eye ($P = 0.83$, Fig. 6B), and between the left/right hemisphere surface ratio and the total retinal thickness of the left eye ($P = 0.61$, Fig. 6C).

### DISCUSSION

This study demonstrates that rat pups exposed to HI at P10 developed a retinopathy. Hypoxia-ischemia induced a functional impairment of the inner retina (i.e., the layers of cells connecting the photoreceptors to the brain) while sparing the photoreceptor function, as demonstrated by a severely attenuated b-wave (i.e., activity of the inner retina) in the presence of a well-preserved a-wave (i.e., activity of the photoreceptors) of the mixed rod-cone ERG responses. Rod- and cone-mediated inner retinal functions were compromised, as was demonstrated by the attenuated rod-mediated response (rod $V_{\text{max}}$) and photopic b-wave. The histology of these eyes confirmed that the RPE and the photoreceptor layers (i.e., the OS, IS, and ONL) were intact, compared to the significantly damaged inner retina (i.e., the OPL, INL, IPL, and RGC/FL). We also confirmed the presence of cerebral damage associated with impaired cerebral visual function as demonstrated by the reduced VEP P100 amplitude and injury on the left hemisphere on brain histology. Interestingly, however, the severity of retinal and cerebral injuries did not correlate, suggesting that visual impairments following HI could arise either from retinal injury, cerebral injury, or a combination of both. This is an important finding, since much of the emphasis until now has been placed on the abnormalities in the intracerebral visual pathways to explain the visual deficits in human newborns with neonatal encephalopathy.$^5$-$^11$ To our knowledge, this is the first demonstration of such risk in a rat model of term neonatal HIE. Previous animal studies have highlighted the risk of the retinopathy following HI, but in younger animals (i.e., at ages that correspond to human preterm infants in whom retina is well known to be susceptible to damages).$^{10-20}$ Kiss et al. reported a reduction in the thickness of the ONL and INL as well as ganglion cell loss in 6-week-old rat retinas following 15 minutes of asphyxia at P0. In another study using a similar rat

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**TABLE 1. Electroretinogram Amplitudes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Severe HI, $n = 6$</th>
<th>Moderate HI, $n = 5$</th>
<th>Mild HI, $n = 4$</th>
<th>Ischemia, $n = 6$</th>
<th>Control, $n = 6$</th>
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<td>P30</td>
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<td>OS</td>
<td>Mixed rodcone a-wave</td>
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$^*$ $P < 0.05$, $^{†} P < 0.001$ versus control.
model, 20 minutes of asphyxia at P0 induced abnormal structural changes in the RGC/FL at P60, marked by neurodegeneration, neovascularization, and gliosis. A 2-hour exposure of P1 rats to 5% oxygen resulted in cell death in the INL and ganglion cell layer, Müller cell swelling, and increased permeability of the retinal blood vessels. Of interest, Huang et al. used the Vannucci model at P7 and demonstrated substantial inner retinal damage (with apoptosis) and gliosis accompanied by a reduction in the ERG b-wave amplitude at P14–60, consistent with our findings. However, they did not report a complete destruction of the IPL and RGC/FL as observed in the severe HI group in our study. The discrepancies between different studies may represent a strain difference in retinal susceptibility to HI, as similar strain difference has been reported in the oxygen-induced retinopathy model. Alternatively, the discrepancies may be due to the difference in

**Figure 2.** Neonatal HI–induced functional impairments of the ipsilateral retina at postnatal day 60 (P60). (A) Representative scotopic (mixed rod-cone and rod V_max) and photopic ERGs. Horizontal and vertical calibrations in milliseconds (ms) and μVolts (μV), respectively. Vertical arrows correspond to stimulus onset. The a-wave and the b-wave are indicated by a and b, respectively. The ERGs from the left eye (i.e., ipsilateral to the carotid ligation, OS; solid line) of the HI animals remained in the same subgroups as initially categorized at P30. The ERGs from the right eyes (i.e., contralateral to the carotid ligation, OD; dotted line) of the HI animals appeared similar to those of the left eyes of the control animals. (B) Amplitude measurements of the ERG waves. Clear bars, left eyes (OS); shaded bars, right eyes (OD). Mean ± SD. Hypoxia-ischemia subgroups compared to the control group: *P < 0.05. No significant difference was found in the scotopic a-wave amplitude between the groups. The amplitudes of the mixed rod-cone b-wave and rod V_max were significantly decreased in the left eyes of the moderate and severe HI groups, but not in the left eyes of the mild HI group and the right eyes of all HI animals. The amplitude of the photopic b-wave was significantly decreased in the left eyes of the moderate and severe HI groups, and increased in the right eyes of the mild HI group, but was not different in the other groups.
the age at which HI was induced. The neurodevelopmental stage of a P0 rat is equivalent to that of very preterm infant (24 weeks gestational age), P7 is equivalent to late-preterm (32–36 weeks gestational age), whereas P10 is considered closer to full-term (40 weeks gestational age). 26

The degree of the abnormalities in the ERGs and histology of the HI animals varied. We also noted a variability in the degree of brain injury, at the functional and structural level, and that has been attributed previously, at least in part, to the interindividual difference in the number or efficiency of collaterals in the Vannucci model.21,38 Interestingly, however, the severity of retinal injury and cerebral injury did not correlate, suggesting that retinal and cerebral injuries may occur separately. Differences in local compensatory mechanisms, such as hemodynamics, may underlie this phenomenon. A significant interindividual variability in outcome is evident also in human term newborns with neonatal encephalopathy.39 Further investigations are needed to better understand the factors contributing to the individual differences in retinal susceptibility to HI.

The inner retina appeared more damaged at the center and the far periphery compared to the midperiphery, which was observed in the superior and inferior retinas. This finding suggests that some regions of the retina are more susceptible to HI compared to others. The topographic distribution of injury may arise from the regional differences in blood circulation.40

FIGURE 3. Neonatal HI–induced structural damages to the left retina at postnatal day 60 (P60). (A) Representative toluidine blue stained retinal cross sections from the left eyes (magnification, ×40). Images were taken at approximately 1000 µm from the optic nerve head in the inferior retina. Variability was present in the severity of the injuries to the inner retina in the left eyes (i.e., ipsilateral to the carotid ligation) of the different HI subgroups, while the outer retina appeared relatively intact, compared to the control. (B) Thickness of the retinal layers from the left eyes. Mean ± SD. Hypoxia-ischemia subgroups compared to the control group: *P < 0.05; **P < 0.01; ***P < 0.001. The thickness of the total retina, OPL, INL, IPL, and RGC/FL were significantly decreased in the moderate and severe HI groups, but not in the mild HI group, compared to the control. The severe HI group showed an increase in OS, IS, and ONL thicknesses.
Retinal blood flow is higher in the central region, explaining why retinal cells in the central region may be more sensitive to a decrease in blood flow. In rats, the formation and remodeling of the retinal vasculature progresses from the center toward the periphery and from the superficial (at the level of the FL) to the deep plexus (at the level of the INL) between birth and the third postnatal week. The remodeling period is terminated once the newly formed blood vessels acquire a pericyte coating. At P10, the superficial plexus fully covers the retina, with pericytes around its arterioles and primary branches, whereas a capillary-free zone forms. In contrast, the deep plexus does not reach the edge of the retina until around P14. The capillary-free zones around the optic disc may also explain the more pronounced damage to the central part of the retina compared to the midperiphery. The fact that the far periphery still is not vascularized by the deep plexus and remains pericyte-free at P10 may provide an additional explanation as to why the far periphery was more damaged than the midperiphery. More investigations are needed to understand the changes in retinal blood vessel architecture following HI and how these changes vary with eccentricity. This may help elucidate the mechanisms involved in the development and progression of the retinopathy that follows HI at term.

The abnormalities in retinal function and structure could only be obtained if the rat pups were subjected to hypoxia following the unilateral carotid ligation, which emphasizes the crucial role played by the hypoxic event in addition to ischemia. One of the limitations of our study is a lack of true hypoxia-only control (hypoxia without carotid ligation). Previous studies showed that when hypoxia and ischemia were used separately, neither produced measurable cerebral injury. This is not to say that the retina would react the same way as the brain in the presence of an ischemic or hypoxic event. However, a near absence of meaningful results from the contralateral eye (hypoxia-only retina) of the experimental group (ligation + hypoxia) or the ligation-only group suggests a minimal (if any) effect of hypoxia to the retinal structure and function at least until P60. Some contralateral eyes (the severe group at P30 and the mild group at P60), however, did show a slight yet significant supranormal ERG amplitudes and this may represent such an effect, as it was previously shown that ischemia could yield supranormal ERGs in the ischemic or the fellow eye.

The selective resistance of the photoreceptors to HI compared to the inner retina is intriguing especially since the photoreceptors are the cells that consume the most oxygen per gram of tissue weight in the body. It may be explained by the high mitochondrial content of the photoreceptors that is needed to provide enough energy to meet their high metabolic demands. Hypoxia has been shown to induce an enlargement of the mitochondria, which results in an increased resistance to apoptosis and survival. Furthermore, the photoreceptors are rich in neuroglobin, an oxygen-binding globin protein, which is known to have a neuroprotective role. Photoreceptors have been shown to have a better ability to reach their metabolic demand anaerobically compared to the inner retina. Photoreceptors also are more resistant to glutamate excitotoxicity, which is known to be one of the main mechanisms underlying ischemic retinal injury. The selective resistance of the photoreceptors may finally arise from the differences in the blood supplies between the photoreceptors and the inner retina. The photoreceptors are perfused by the choroidal artery and the choriocapillaries, which receive approximately 80% of the total blood supply to the eye, but have low oxygen extraction; the inner retina is perfused by the branches of the retinal artery, which receives only 20% of the total blood supply to the eye, but has high oxygen extraction. Integrity of the blood-retinal barrier is better preserved on the choroidal vasculature side compared to the retinal vasculature side after an HI. Further investigations are needed to determine which of the above mentioned hypotheses is/are correct. Also, a long-term follow-up on retinal function and structure following neonatal HI is needed to rule out the possibility of a slower degeneration of the photoreceptors that is not yet evidenced at P60.

One could argue that a bilateral common carotid occlusion, which produces symmetrical brain injuries in rats, may have been a more representative model of the brain injuries encountered in human term asphyxiated newborns, since these injuries usually are bilateral and symmetrical. However, for this study, we chose the unilateral model so to examine the role of HI in the development of brain and retinal injuries, while also taking into account the role of reperfusion in the overall outcome. In addition, since the brain injury is located in the left hemisphere and more than 90% of the retinal fibers cross over at the optic chiasm to the opposite side of the brain in rats, studying the right eye in this model allowed us to distinguish visual impairments arising from the retina versus the brain. In our model, the visual pathway driven by the left eye was impaired due to reduced retinal output, whereas the visual pathway driven by the right eye was most probably impaired due to cerebral anomaly, given the absence of functional deficit at the retinal level. Thus, the animal model combining a unilateral carotid ligation with a 2-hour exposure to hypoxia in P10 rat pups gave us a unique opportunity to study the different impacts of cerebral versus retinal injuries on vision.

### Table 2. Thicknesses of the Retinal Layers

<table>
<thead>
<tr>
<th>Layer</th>
<th>Control, n = 8</th>
<th>Mild HI, n = 3</th>
<th>Moderate HI, n = 5</th>
<th>Severe HI, n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total thickness, μm</strong></td>
<td>214.79 ± 26.03</td>
<td>197.75 ± 13.13</td>
<td>152.59 ± 19.68‡</td>
<td>141.31 ± 18.46‡</td>
</tr>
<tr>
<td>RPE thickness, μm</td>
<td>7.97 ± 0.19</td>
<td>7.75 ± 0.93</td>
<td>8.20 ± 0.41</td>
<td>8.57 ± 1.15</td>
</tr>
<tr>
<td>OS thickness, μm</td>
<td>28.47 ± 6.93</td>
<td>28.10 ± 4.39</td>
<td>33.48 ± 5.97</td>
<td>40.98 ± 9.07‡</td>
</tr>
<tr>
<td>IS thickness, μm</td>
<td>13.86 ± 2.80</td>
<td>12.15 ± 3.11</td>
<td>15.50 ± 2.03</td>
<td>21.09 ± 3.20‡</td>
</tr>
<tr>
<td>ONL thickness, μm</td>
<td>47.64 ± 6.75</td>
<td>40.05 ± 7.50</td>
<td>51.13 ± 5.93</td>
<td>57.20 ± 5.68‡</td>
</tr>
<tr>
<td>OPL thickness, μm</td>
<td>10.00 ± 2.23</td>
<td>9.86 ± 4.20</td>
<td>6.16 ± 1.36‡</td>
<td>6.00 ± 0.00‡</td>
</tr>
<tr>
<td>INL thickness, μm</td>
<td>28.93 ± 5.22</td>
<td>24.59 ± 2.18</td>
<td>16.35 ± 4.23‡</td>
<td>9.77 ± 1.39‡</td>
</tr>
<tr>
<td>IPC thickness, μm</td>
<td>54.99 ± 10.34</td>
<td>56.78 ± 13.58</td>
<td>19.79 ± 9.61‡</td>
<td>2.85 ± 3.41‡</td>
</tr>
<tr>
<td>RG/C/FL thickness, μm</td>
<td>22.92 ± 5.35</td>
<td>19.39 ± 8.94</td>
<td>7.54 ± 10.93†</td>
<td>0.84 ± 1.87†</td>
</tr>
</tbody>
</table>

* P < 0.05.  † P < 0.01.  ‡ P < 0.001 versus control.
The maturity of the rat retina at P10 may be slightly behind that of humans at term. Although most retinal layers are present at P10, the first measurable ERGs do not appear until approximately P12,62 which then matures up to P30.33 Although the human retina is functional at term, it also undergoes rapid structural and functional maturation during first 3 to 4 months.63–66 In addition, the visual system refines its connections through visual inputs up to 3 years of age. Another difference between the human and rat retinas is that humans have higher percentage of cones in comparison with rats, and the distribution of the cones is highly concentrated in the fovea within the macula, while the rats do not have a macula. The blood supplies to the retina are similar in humans and rats. The outer retina is supplied by the choroidal vasculature (middle and posterior ciliary arteries), whereas the inner retina is supplied by the retinal vasculature (central retinal artery), with a similar developmental sequence (the choroidal circulation before the retinal circulation, center to periphery, superficial to deep).
Further investigations thus are needed to determine if human asphyxiated term newborns develop similar functional and structural damages to the retina as observed in this study in the rat model. The results observed in the rat model may not be generalizable to human newborns with neonatal encephalopathy.

In conclusion, we found functional and structural anomalies in the retina following HI with or without the presence of brain injury. Specifically, these injuries were limited to the inner retina, while the photoreceptors were relatively spared. These findings suggest that retinal injuries, in addition to or independent of cerebral injuries, may contribute to visual impairments in term asphyxiated newborns, and warrant further studies in humans and animals to better understand the disease process underlying the retinal damages associated with neonatal asphyxia and their relation to brain injury. Distinguishing retinal injuries from cerebral injuries is a worthy challenge that will help with the planning of improved treatments for these newborns.

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References


43. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development. 1998;125:1591–1598.
47. Henkes HE. Electroretinography in circulatory disturbances of the retina. II. The electroretinogram in cases of occlusion of the central retinal artery or of its branches. AMA Arch Ophthalmol. 1954;51:42–53.