Vulnerability of Dopaminergic Amacrine Cells and Optic Nerve Myelination to Prenatal Endotoxin Exposure

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PURPOSE. Intrauterine infection has been linked to preterm delivery and neurologic injury. The purpose of this study was to investigate the effects of fetal inflammation induced by exposure to endotoxin on the structure and neurochemistry of the retina and optic nerve.

METHODS. The bacterial endotoxin, lipopolysaccharide (LPS), was administered to fetal sheep at ~0.65 of the ~147-day gestation period via repeated bolus doses (1 µg/kg per day) over 5 days, with fetal retinas and optic nerves assessed 10 days after the first LPS exposure.

RESULTS. In the retina, the total number of tyrosine hydroxylase immunoreactive (TH-IR), dopaminergic amacrine cells was reduced (P < 0.05) in LPS-exposed compared with control fetuses. There was no difference in the number of ChAT-, substance P-, or NADPH-d-positive amacrine cells. The total number of myelinated axons in the optic nerve was not different (P > 0.05) between groups; however, the myelin sheath was thinner (P < 0.05) in LPS-exposed fetuses.

CONCLUSIONS. Prenatal exposure to repeated doses of endotoxin results in alterations to the retina and optic nerve with specific effects on dopaminergic neurons and myelination, respectively. These findings could have implications for visual function. (Invest Ophthalmol Vis Sci. 2007;48:472–478) DOI: 10.1167/iovs.06-0709

Intrauterine infection may occur as a result of maternal urinary tract infections, wounds, chorioamnionitis, vaginitis, and septicemia or may even be fetal in origin. Such infections have been causally linked to preterm birth and very low birth weight (VLBW) and have been shown to increase the risk of major neurologic disorders such as cerebral palsy. It is possible that prenatal infection may also be associated with more subtle abnormalities of the central nervous system including alterations to the visual system. The developing visual system is of interest because severely preterm infants have an increased risk of long-term visual impairments; these include reduced visual acuity and refractive errors and subtle changes including abnormalities in color vision and contrast sensitivity.

Our objective was to investigate the effects on the developing retina and optic nerve of prenatal exposure to the bacterial endotoxin lipopolysaccharide (LPS), a potent inducer of inflammation. We exposed fetal sheep to LPS over 5 days, commencing at 0.65 of gestation (term ~147 days); this fetal age was chosen as it equates to 25 weeks of gestation in the human fetus, a period of increased vulnerability to brain injury. In previous studies using this model, we have shown that fetal exposure to LPS leads to white-matter injury ranging from diffuse subcortical damage to periventricular leukomalacia. The ovine fetus is an ideal model for examining clinically relevant experimental paradigms such as intrauterine infection on retinal development, as the sheep has a long gestation period and a sequence of in utero retinal development similar to that of humans. In the retina at 0.65 of gestation, all neuronal populations of amacrine cells including tyrosine hydroxylase immunoreactive-(TH-IR) dopaminergic, cholinergic (ChAT), and substance P-containing cells. The effects on dopaminergic amacrine cells, which are believed to play a role in contrast sensitivity, were of particular interest, as these cells are known to be affected both pre- and postnatally by placental insufficiency in sheep and guinea pigs. In addition, we performed quantitative analyses to assess the effects of LPS exposure on the retinal vasculature, optic nerve myelination, and astrocyte and axon numbers; the latter indicates ganglion cell number.

METHODS

Surgical Preparation

Our study received institutional approval and conforms to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the National Health and Medical Research Council of Australia code of practice. Aseptic surgery was performed at 91.0 ± 0.3 days after mating (term ~147 days of gestation). In 12 pregnant ewes, carrying single fetuses, for the implantation of catheters into the fetal femoral artery (for blood sampling) and vein (for LPS injection) and amniotic sac. Antibiotics (procaine penicillin, 200 mg/mL and dihydrostreptomycin, 250 mg/mL; Invet, Bendigo, Victoria, Australia) were administered intramuscularly to the fetus. After surgery, the sheep were held in individual cages with ad libitum access to food and water.

Experimental Protocol

A protocol of repeated injections of LPS was used, as preliminary observations indicated that single injections of a sublethal dose were...
ineffective in causing cerebral white matter damage,\textsuperscript{15} a crucial criteri-
on in making this a clinically relevant model of intrauterine infection.

Beginning at 95 dg, intravenous boluses of LPS (1 μg/kg; \textit{Esche-
richtia coli}, 055:B5; Sigma-Aldrich, St. Louis, MO) were administered to six
ovine fetuses over 5 days. Four fetuses received five daily
injections (5 μg in total); two received three injections on alternate
(days (3 μg in total) as their arterial oxygen saturation (SaO\textsubscript{2}) had not
returned to preexposure levels within 24 hours.\textsuperscript{15} Fetal arterial blood
gas status was sampled twice hourly for 8 hours after LPS administra-
tion. Catheterized control fetuses (\(n = 6\)) received daily injections of
saline between 95 and 99 dg. In addition, two nonsurgical control
fetuses were euthanized at 95 dg to assess optic nerve development
at the time of initial LPS-exposure.

\section*{Tissue Preparation}

Fetuses and ewes were euthanized with an overdose of pentobarbitone
sodium (Lethobarb; 130 mg/kg, IP, Virbac Animal Health, Peakhurst,
NSW, Australia) at 105 dg. The fetal eyes were perfused in situ with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), enucleated,
and immersion-fixed overnight in 4% PFA in 0.1 M PB.

\section*{Retina}

\subsection*{Preparation.}

Retinas were dissected and processed as previ-
ously described.\textsuperscript{18} The left retinas were prepared as wholemounts for
TH-IR. From the right retinas, small blocks of tissue (2 × 5 mm) were
collected both centrally (immediately adjacent to the optic nerve head,
temporal central [TC]); and peripherally (from the inferior temporal
quadrant, temporal peripheral, [TP]) and embedded in either paraffin
or epoxy-impregnated, and immuno-processed with the avidin-biotin
peroxidase complex (Vector Laboratories, CA). Immunohistochemical
antibodies to anti-ChAT (1:1000; Chemicon International, Temecula, CA),
at anti-substance P (1:1000; BD Biosciences, San Diego, CA); and mouse anti-
TH (1:1000; Chemi-
con International) were used to identify amacrine cell subpopulations,
and rabbit anti-GFAP (1:500; Sigma-Aldrich) and mouse anti-CNPase (1:100;
Sigma-Aldrich, St. Louis, MO) were performed using the avidin-biotin peroxidase
complex to identify macrophages/reactive microglial cells.

\subsection*{Immunohistochemistry.}

\textbf{Antibodies to anti-ChAT (1:1000; Chemicon International, Temecula, CA),
at anti-substance P (1:1000; BD Biosciences, San Diego, CA); and mouse anti-
TH (1:1000; Chemi-
con International) were used to identify amacrine cell subpopulations,
and rabbit anti-GFAP (1:500; Sigma-Aldrich) were used to identify astro-
cytes with the avidin-biotin peroxidase complex (Vector Laboratories,
Burlingame, CA).\textsuperscript{18} All material was stained simultaneously for each
marker to avoid procedural variation, and omitting the primary anti-
bodies resulted in no staining.

\subsection*{Histology.}

Biotinylated \textit{Lycopersicon esculentum} (to-
mato) lectin (1:250; Sigma-Aldrich) histology was used to visual-
ize macrophages/reactive microglia as previously described.\textsuperscript{23}

\subsection*{NADPH-d Histochemistry.}

Neuronal nitric oxide synthetase
(nNOS)-containing nitrergic amacrine cells also stain with NADPH-d
histochemistry in sheep.\textsuperscript{19} We used this latter technique to identify
NADPH-d (ND)\textsubscript{1} and ND\textsubscript{3} populations of nitrergic amacrine cells in the
NI quadrant of the right retina.

\section*{Qualitative Analysis}

\subsection*{Gliosis.}

Qualitative assessment was performed in 8-μm transverse
sections of the retina reacted for GFAP-IR, to determine whether there
was any difference in the extent or intensity of staining between
LPS-exposed and control fetuses. From each animal, three transverse
sections of both peripheral and central retina were examined.

\subsection*{Macrophages/Activated Microglia.}

Lectin-stained trans-
verse 8-μm sections from LPS-exposed and control fetuses were exam-
ined qualitatively for the presence of lectin-positive macrophages/
reactive microglial cells. As before, three transverse sections of both
peripheral and central retina were examined from each animal.
Histochemistry. Biotinylated *Lycopersicon esculentum* (tomato) lectin (1:250; Sigma-Aldrich) histochemistry was used to visualize macrophages/reactive microglia as previously described.\textsuperscript{25}

Quantitative Analysis

Control tissue at 95 dg was examined to determine whether myelination was occurring at the gestational age at which LPS was first administered. LPS-exposed and control tissue was examined at 105 dg for the presence of lectin-positive macrophages/reactive microglial cells.

**Qualitative Analysis**

There was no difference in total body or brain weight between groups (Table 1). There were no differences in total retinal areas between LPS-exposed and control fetuses (Table 1). There were also no differences (\(P > 0.05\)) in the proportion of retinal areas occupied by blood vessels between LPS-exposed (17.2% ± 1.0%) and control retinas (16.6% ± 1.5%). There was no evidence of new vessel formation or alteration to blood vessel morphology.

**Retinal Morphology**

**Structure.** In control retinas at 105 dg, mitosis has ceased, and all retinal layers were present. Apoptotic cells were occasionally observed in the ONL. Photoreceptor inner segments were present but outer segments were only just beginning to develop. No gross morphologic alterations were observed in the cytoarchitecture of the retina in LPS-exposed compared with control fetuses. There were also no differences (\(P > 0.05\)) in the thickness (total or individual layers) of the central (total: LPS-exposed, 231 ± 11 mm versus control, 260 ± 14 mm) or peripheral (total: LPS-exposed, 222 ± 13 mm versus control, 223 ± 10 mm) retina between LPS-exposed and control fetuses. There were no differences in total retinal areas between LPS-exposed and control fetuses (Table 1).

**Ganglion Cell Somal Areas.** There were no differences in the somal areas of ganglion cells between LPS-exposed (193 ± 13 mm\(^2\)) and control fetuses (200 ± 10 mm\(^2\)).

**Vasculature.** There were no differences in the proportion of retinal vessels occupied by blood vessels between LPS-exposed (17.2% ± 1.0%) and control retinas (16.6% ± 1.5%). There was no evidence of new vessel formation or alteration to blood vessel morphology.

**Cell Populations and Neurochemistry**

**TH-IR Amacrine Cells.** In control retinas, the total number (\(P < 0.005\)) of TH-IR amacrine cells was reduced in LPS-exposed compared with control fetuses (Table 1). However, the somal areas of TH-IR amacrine cells were not different between groups (Table 1). No differences were observed in the extent of injury to TH-IR amacrine cells that received three doses versus five doses of LPS, with respect to any of the parameters assessed in the present study.

**NADPH-d Amacrine Cells.** There were no differences (\(P > 0.05\)) in the total number or density of ND1 or ND3 NADPH-d-positive amacrine cells in LPS-exposed compared with control fetuses (Table 1). There were no differences (\(P > 0.05\)) in the somal areas of ND1 or ND3 amacrine cells between groups (Table 1).

**Substance P-IR Amacrine Cells.** The total number (\(P > 0.05\)) and density (\(P > 0.05\)) of Substance P-IR amacrine cells were not different between LPS-exposed and control fetuses (Table 1).

**Table 1. Analysis of Amacrine Cell Populations**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS-Exposed</th>
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<tbody>
<tr>
<td>Total retinal area (mm(^2))</td>
<td>643.0 ± 13.5</td>
<td>631.67 ± 32.1</td>
</tr>
<tr>
<td>Total TH-IR cell number (cells/retina)</td>
<td>10,369 ± 683</td>
<td>6,603 ± 603*</td>
</tr>
<tr>
<td>TH-IR cell density (cells/mm(^2))</td>
<td>16 ± 1</td>
<td>11 ± 1*</td>
</tr>
<tr>
<td>TH-IR somal area (μm)</td>
<td>91 ± 6</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>TH-IR dendrites/soma</td>
<td>2.0 ± 0.2</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>Total TH-IR dendrite length (μm)</td>
<td>35.6 ± 8.2</td>
<td>16.1 ± 0.9†</td>
</tr>
<tr>
<td>Total ND1 cell number (cells/retina)</td>
<td>64,265 ± 4,557</td>
<td>53,956 ± 2,451</td>
</tr>
<tr>
<td>ND1 cell density (cells/mm(^2))</td>
<td>105 ± 7</td>
<td>87 ± 7</td>
</tr>
<tr>
<td>ND1 somal area (μm)</td>
<td>98 ± 3</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>Total ND3 cell number (cells/retina)</td>
<td>97,761 ± 5,242</td>
<td>87,416 ± 5,015</td>
</tr>
<tr>
<td>ND3 cell density (cells/mm(^2))</td>
<td>160 ± 9</td>
<td>143 ± 11</td>
</tr>
<tr>
<td>ND3 somal area (μm)</td>
<td>41 ± 2</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>Total Substance P cells (cells/retina)</td>
<td>117,521 ± 24,651</td>
<td>117,468 ± 10,939</td>
</tr>
<tr>
<td>Substance P cell density (cells/mm(^2))</td>
<td>194 ± 39</td>
<td>190 ± 19</td>
</tr>
<tr>
<td>Substance P somal area (μm)</td>
<td>29 ± 2</td>
<td>28 ± 1</td>
</tr>
</tbody>
</table>

Data are the mean of means ± SEM. \(n = 6\), control; \(n = 6\), LPS-exposed fetuses.

\* \(P < 0.005\).

\† \(P < 0.01\).

\textsuperscript{25} A. Loeliger et al., JOVS, January 2007, Vol. 48, No. 1.
ChAT-IR Amacrine Cells. There were no overt differences in the morphology or density of ChAT-IR amacrine cells in LPS-exposed compared with control fetuses. This reduction is illustrated by comparing density plots (A, control) and (B, LPS-exposed). Symbol size in the adjacent key is proportional to density per square millimeter. (C, D) Photomicrographs of TH-IR retinal.wholomounts showing a reduction in the number of cells (arrow) in LPS-exposed (D) compared with control fetuses (C). Insets: reduction in the number of dendrites and length in LPS-exposed (D, inset) and control (C, inset) fetuses. Scale bars: (A, B) 7 mm; (C, D) 220 μm; (C, D inset) 60 μm.

**DISCUSSION**

Repeated exposure of the ovine fetus to LPS at 0.65 of gestation resulted in alterations to dopaminergic amacrine cells in the retina and to myelination in the optic nerve when examined 10 days after the initial exposure. These changes were not associated with overt lesions, astrogliosis, structural alterations or evidence of infiltrating macrophages/activated microglia. It is possible that microglial invasion occurred transiently before the endpoint of this experiment; in the rat it has been reported that LPS exposure causes an acute inflammatory response within 48 to 72 hours, attenuating by 6 days after insult. We have previously shown that repeated LPS exposure results in systemic hypoxemia, hypotension, and increased plasma levels of the pro inflammatory cytokine, IL-6, indicative of a systemic inflammatory response. Hence, the alterations to retinal neu-
riones and to myelination that we have observed could have been caused by either retinal hypoxia/underperfusion, exposure to proinflammatory cytokines and/or other as yet unidentified factors.

Effects of LPS Exposure on Retinal Amacrine Cells

Repeated fetal exposure to LPS had detrimental effects on the morphology and number of dopaminergic amacrine cells in the retina. This was not a global effect on all retinal amacrine cells as cholinergic, nitricergic, and substance P-containing amacrine cells were unaffected. This reduction could be due to either dopaminergic amacrine cell loss or a downregulation of TH; both have the potential to cause alterations of function.

Adverse effects on dopaminergic amacrine cells are of particular interest, since alterations to the dopaminergic system are thought to affect contrast sensitivity, which has been shown to be altered in infants born prematurely. Dopaminergic amacrine cells are first observed in fetal sheep retina at 72 dg and are thus well established at the onset of LPS exposure. The associated retinal hypoxemia and hypotension, and increased levels of proinflammatory cytokines (IL-6), could affect cell proliferation, cell survival or cause the downregulation of TH expression.

It is of interest that neonatal intracerebral and intranigral injections of LPS in the adult rat result in a significant loss of TH-IR neurons; this loss is maintained 1 year after injection in the adult. Studies in the rat support the role of inflammatory processes, mediated by microglial production of inflammatory cytokines and nitric oxide, in causing damage to dopaminergic neurons in the substantia nigra.

We have now demonstrated that dopaminergic amacrine cells are vulnerable to two forms of prenatal insult—endotoxin exposure in sheep and chronic placental insufficiency in both guinea pigs and sheep—confirming the high vulnerability of this class of cells. It is possible that other inflammatory agents such as tumor necrosis factor (TNF)-α and interferon (INF)-γ, which increase IL-6 levels and lead to inflammation in the adult retina, may induce similar changes in the developing visual system.

As indicated, it has been proposed that dopaminergic amacrine cells may have a role in contrast sensitivity, and we have reported evidence for functional changes in these cells in the guinea pig by detecting alterations in the ERG. This finding is potentially important, as contrast sensitivity is impaired in children born very prematurely.

Effect of LPS Exposure on Retinal Thickness

There was no difference in retinal thickness (total or individual layers) after LPS exposure, indicating that overall there is no major neuronal loss or alterations to process development at 105 dg. This does not exclude the possibility that LPS-exposure may have long-lasting effects on connectivity, as process growth in dopaminergic amacrine cells was reduced. Photoreceptors are immature at the time of LPS administration (95 dg) with inner segments first observed at 100 dg and outer segments not beginning to develop until 105 dg (Loeliger M, unpublished observations, 2006). Although there is no evidence of photoreceptor changes within the timeframe of the present study, follow up studies would be valuable in indicating whether damage was sustained in the long term. We know that photoreceptors are vulnerable to hypoxic-ischemic insults and can be affected by chronic hypoxemia in the ovine fetus.

There was no difference in the somal areas of retinal ganglion cells or in the number of myelinated optic axons between groups, indicating that ganglion cell numbers and morphology are not affected by LPS. It is possible that populations of retinal neurons not assessed in the present study, including horizontal cells, which are vulnerable to placental insufficiency in the guinea pig, may also be affected by LPS.

Retinal Neovascularization

Retinal hypoxia-ischemia is a central feature in diseases in which retinal neovascularization occurs, including retinopathy of prematurity and diabetic retinopathy (see review in Ref. 44). No evidence of neovascularization or alterations in the proportion of the retina occupied by blood vessels was observed after LPS exposure within the timeframe of the experiment. Low levels of insulin-like growth factor (IGF)-1 in premature infants and increases in vascular endothelial growth factor (VEGF) have been implicated in the etiology of retinopathy of prematurity. Investigation of both IGF-1 and VEGF levels after LPS exposure would be of interest and may provide insight into the possibility of vascular changes developing in the long term.

Effect of LPS Exposure on Optic Nerve Development

Fetal cerebral white matter is particularly sensitive to hypoxia during development. Injury of the cerebral white matter of LPS-exposed fetuses ranged from diffuse subcortical damage to periventricular leukomalacia. Although no overt lesions were observed in the optic nerve of LPS exposed fetuses, the thickness of the myelin sheath was reduced. LPS exposure occurred at a critical time for optic nerve development, as myelination has not yet commenced at 95 dg and <10% of a total population of approximately 1 million axons observed in the adult sheep optic nerve are myelinated at the completion of the study (105 dg).

CNPase-IR is a marker for the early myelinating phase of the oligodendrocyte lineage and positive cells form a significant
proportion of the oligodendrocyte population at this gestational age. As the number of CNPase-IR oligodendrocytes was not affected in LPS-exposed fetuses, the decrease in myelin is unlikely to be due to a reduction in the number of myelinating oligodendrocytes. The capacity of the oligodendrocytes to produce myelin, however, appears to be altered, and this may affect conduction velocity and neural function. We have previously shown myelination to be altered by placental insufficiency in fetal sheep and guinea pigs. Examination of the optic nerve at a later time point would give a clearer picture as to whether bolus LPS-exposure causes permanent alterations to myelination or whether this process has been only transiently retarded, as occurs in the ovine fetus after placental insufficiency in late gestation or repeated exposure to betamethasone. There was no difference in the optical density of GFAP-IR between groups indicating that no astrocytic response had occurred over the period of the investigation.

Implications for the Visual System

Although there was no overt damage in the retina or optic nerve 10 days after initial exposure to LPS, examination closer to term or in the postnatal period may reveal alterations to cell numbers, dendritic outgrowth, and synaptogenesis not evident in the short term. Long-term studies would also show whether the changes in dopaminergic amacrine cells or optic nerve myelination after repeated LPS exposure are permanent and have the potential to cause functional deficits after birth. Although the alterations observed in this study were subtle, they have important physiological implications. Reduced vision has significant psychosocial and socioeconomic implications and any evidence that may help to elucidate the underlying etiology and potential mechanisms of the increased incidence of visual abnormalities in preterm infants is clinically important for neonatal care and ongoing support for these individuals.

Conclusions

Repeated fetal exposure to an inflammatory insult results in alterations in the retina and optic nerve, including a reduction in dopaminergic amacrine cell numbers and in the thickness of myelin in the optic nerve. These changes have the potential to cause alterations to visual function after birth.

References