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Gestational Lead Exposure Selectively Decreases Retinal Dopamine Amacrine Cells and Dopamine Content in Adult Mice

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Abstract

Gestational lead exposure (GLE) produces supernormal scotopic electroretinograms (ERG) in children, monkeys and rats, and a novel retinal phenotype characterized by an increased number of rod photoreceptors and bipolar cells in adult mice and rats. Since the loss of dopaminergic amacrine cells (DA ACs) in GLE monkeys and rats contributes to supernormal ERGs, the retinal DA system was analyzed in mice following GLE. C57BL/6 female mice were exposed to low (27 ppm), moderate (55 ppm) or high (109 ppm) lead throughout gestation and until postnatal day 10 (PN10). Blood [Pb] in control, low-, moderate- and high-dose GLE was ≤ 1 , ≤ 10 , ~ 25 and ~ 40 $\mu\text{g/dL}$, respectively, on PN10 and by PN30 all were ≤ 1 $\mu\text{g/dL}$. At PN60, confocal-stereology studies used vertical sections and wholemounts to characterize tyrosine hydroxylase (TH) expression and the number of DA and other ACs. GLE dose-dependently and selectively decreased the number of TH-immunoreactive (IR) DA ACs and their synaptic plexus without affecting GABAergic, glycinergic or cholinergic ACs. Immunoblots and confocal revealed dose-dependent decreases in retinal TH protein expression and content, although monoamine oxidase-A protein and gene expression were unchanged. High-pressure liquid chromatography showed that GLE dose-dependently decreased retinal DA content, its metabolites and DA utilization/release. The mechanism of DA selective vulnerability is unknown. However, a GLE-induced loss/dysfunction of DA ACs during development could increase the number of rods and bipolar cells since DA helps regulate neuronal proliferation, whereas during adulthood it could produce ERG supernormality as well as altered circadian rhythms, dark/light adaptation and spatial contrast sensitivity.

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Conflict of Interest Statement

The authors declare that they have no competing financial interests.

Keywords

Lead; gestational exposure; retina; amacrine cells; dopamine; cell loss

Introduction

Lead is a potent and pervasive environmental neurotoxicant that adversely affects the developing brain and retina. Low-level (blood lead concentration [BPb] ≤ 10 $\mu\text{g/dL}$) to moderate-level ([BPb] 11–39 $\mu\text{g/dL}$) developmental lead exposure produces cognitive, auditory, retinal and visual-motor dysfunction in children and experimental animals (Altmann et al., 1998; Canfield et al., 2003; Fox et al., 1997; Fox and Boyes, 2007; Fox et al., 2008; Giddabasappa et al., 2011; He et al., 2003; Nagpal and Brodie, 2009; Osman et al., 1999; Rothenberg et al., 2000, 2002; Wasserman et al., 2000). Children, adult monkeys and rats with gestational lead exposure (GLE) exhibit novel rod photoreceptor-mediated (scotopic) electroretinogram (ERG) supernormality characterized by increased a-wave and b-wave amplitudes (Fox et al. 2008; Lilienthal et al. 1988, 1994; Nagpal and Brodie 2009; Rothenberg et al. 2002). Moreover, adult rats and mice with GLE have a selectively increased number of late-born rod photoreceptors and bipolar cells (Fox et al., 2008; Giddabasappa et al., 2011).

Adult rats with low-level GLE have decreased retinal dopamine content and utilization/turnover (Fox et al., 2008), whereas adult monkeys with lifetime lead exposure have a decreased number of tyrosine hydroxylase-immunoreactive (TH-IR) dopamine (DA) amacrine cells (ACs) (Kohler et al., 1997). Furthermore, adult mice with low-level GLE have decreased forebrain DA content (Leasure et al., 2008). The authors of both studies note that the loss of DA ACs might contribute to the scotopic ERG supernormality (Lilienthal et al., 1994; Fox et al., 2008) since the selective destruction of DA ACs with the neurotoxin 6-hydroxydopamine produces similar supernormal scotopic ERGs (Oliver et al., 1986; Skrandies and Wässle, 1988). Together, these findings suggest that the murine retinal DA system may be a sensitive target site of low-level GLE.

Retinal DA controls circadian rhythms, gap junction modulation, horizontal cell coupling and rod photoreceptor Na^+ , K^+ -ATPase activity (Kothmann et al., 2009; Ruan et al., 2008; Shulman and Fox, 1996; Witkovsky, 2004). DA production, release and metabolism are initiated by light stimulation (Iuvone et al., 1978; Witkovsky 2004). DA ACs are located in the proximal inner nuclear layer (INL) and send long (0.5 mm) processes laterally in sublamina 1 (S1) of the inner plexiform layer (IPL) and into the outer plexiform layer (Dacey, 1990; Voigt and Wässle, 1987). Retinal DA neurons are postsynaptic to GABAergic ACs, which directly control DA release (Gustincich et al., 1997). The DA cell population makes up less than 1% of all ACs, or approximately 500 cells per mouse retina (Nguyen-Legros et al., 1997).

To determine whether DA ACs in adult mouse retinas were selectively vulnerable to GLE, five experiments were conducted. First, fixed retinal wholemounts and fixed-frozen vertical sections and confocal microscopy determined the density and/or number as well as the localization of TH-IR and dopa decarboxylase (DDC)-IR DA ACs, disabled1 (Dab1)-IR AII glycinergic ACs, GABA-IR ACs and choline acetyltransferase (ChAT)-IR ACs. Second, the protein concentration of TH and ChAT were determined with Western blots. Third, the location and expression level of the DA catabolizing enzyme monoamine oxidase A (MAO-A) along with another mitochondrial protein, cytochrome oxidase IV (COX IV) was examined. Fourth, the expression of TH and MAO-A was examined using real-time quantitative PCR (RT-qPCR). Fifth, high-pressure liquid chromatography (HPLC) was used

to measure the retinal DA content and its two major metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). GLE dose-dependently and selectively decreased the number and density of retinal DA ACs, as there was no change in the number of GABAergic, glycinergic AII or ChAT ACs. Fixed-frozen vertical sections revealed a decrease in the TH-IR plexus throughout the S1 IPL sublamina. Western blots showed a dose-dependent decrease in TH levels with no change in ChAT. Despite decreased TH protein expression, there were no changes in MAO-A protein or gene expression in GLE retinas relative to controls. Finally, GLE dose-dependently decreased retinal DA content and decreased retinal DOPAC and HVA.

Material and Methods

Materials

All chemicals used in these experiments were molecular biology grade and certified lead-free (<5 ppb Pb^{2+}). They were purchased from Sigma-Aldrich Co (St. Louis MO), Fisher Scientific (Pittsburgh, PA) or VWR (West Chester, PA) unless otherwise noted. All primary antibody information (host, epitope, concentration and specificity) is presented in Table 1. All secondary antibodies were obtained from Molecular Probes (Carlsbad, CA).

Mouse model of gestational lead exposure

All experimental and animal care procedures complied with the NIH Public Health Service Policy on the Humane Care and Use of Laboratory Animals (NIH 2002) and were approved by the Institutional Animal Care and Use Committee of the University of Houston. All animals were treated humanely and with regards for alleviation of suffering. The GLE model has been described (Giddabasappa et al., 2011; Leasure et al., 2008). Briefly, C57BL/6 wild-type or tyrosine hydroxylase::red fluorescent protein (TH::RFP) transgenic (Zhang et al., 2004) female mice were exposed to tap water (0 ppm lead) or a low-dose (LD: 27 ppm), moderate-dose (MD: 55 ppm) or high-dose (HD: 109 ppm) of lead acetate in their drinking water for two weeks prior to mating, during mating and pregnancy, and until postnatal 10 (PN10) when the lead solution was removed and replaced with normal tap water. Water, food, and dams' weight as well as offspring measures were monitored throughout pretreatment, mating and pregnancy, after delivery and until tissue collection at PN60 (adult). No significant treatment-related differences were observed (Leasure et al., 2008). Pups were weaned on PN21, and males and females were housed separately until PN60. For this study, [BPb] was determined in one offspring of each control and GLE litter on PN10 and PN30. The [BPb] values were similar to our published values (Leasure et al., 2008). As published (Leasure et al., 2008), control, LD, MD and HD GLE groups had peak [BPb] on PN0 and/or PN10 of 0.72 ± 0.07 , 10.10 ± 0.65 , 27.23 ± 1.39 and 42.06 ± 0.70 $\mu\text{g/dL}$, respectively; on E14 the dam's [BPb] was similar to PN0 pups; and on PN30 the [BPb] in GLE mice were not different from controls. Mice were euthanized by decapitation between 1000–1200 hour. No significant sex differences were observed for any end point, so data for males and females were combined.

Tissue processing for fixed-frozen vertical sections

All tissue processing and immunohistochemistry techniques were performed as described (Fox et al., 2008; Giddabasappa et al., 2011; Johnson et al., 2007). Briefly, enucleated eyes were fixed in buffered 4% paraformaldehyde, cryoprotected in 30% w/v sucrose, embedded with mounting medium, flash frozen in liquid nitrogen and stored at -80°C . Three non-adjacent fixed-frozen transverse sections (10 μm) taken from the vertical meridian of the central retina were mounted on glass slides and stored at -20°C . Thawed slides were post-fixed, blocked and incubated with well-characterized primary antibodies (Table 1). For double- and triple-labeling experiments, primary antibodies from different host animals were

applied simultaneously. Slides were incubated with dilutions of Cy3-, Cy5- or Alexa 488, 555 and 643 conjugated secondary antibodies, dried and mounted with Vectashield (Vector Laboratories Inc., Burlingame CA), coverslipped and stored at 4°C. Fixed-frozen vertical sections were analyzed sequentially for each channel using laser scanning confocal microscopy.

Tissue processing and immunohistochemistry for retinal wholemounts

Following sacrifice, three cuts were made in cleaned neural retinas to flatten them and provide dorsal vs. ventral orientation. Retinas were wholemounted retinal ganglion cell (RGC) side up onto acetate filter paper, placed onto an in-house filter apparatus, and washed while negative pressure was applied. The filter paper was removed and placed into 4% paraformaldehyde for 20 minutes at RT, washed with PBS at RT, transferred to a multi-well plate, and incubated for three hours at RT in 500 μ L of our standard blocking buffer (Giddabasappa et al., 2011; Johnson et al., 2007). After incubation in primary antibodies for seven days, wholemounts were washed with PBS, blocked for one hour at RT, incubated with dilutions of Cy3-, Cy5-, or Alexa 488 conjugated secondary antibodies, washed, then placed on slides, dried and mounted with Vectashield, coverslipped and stored at 4°C. Wholemounts were analyzed sequentially for each channel using confocal microscopy.

Laser scanning confocal microscopy

As described (Giddabasappa et al., 2011; Johnson et al., 2007), confocal images were taken with a Leica TCS SP2 LSCM (Leica Microsystems, Exton, PA). For fixed-frozen vertical sections, stacks of images from different Z-planes. For wholemounts, stacks of images from individual Z-planes were taken from the middle 8 μ m of wholemount. For both, 15–20 separate images were taken with an average step distance of 0.3 μ m per image. Maximum projection images were compiled for analysis to visualize the plexus and cell body in one image. All images were processed minimally with Adobe Photoshop CS software (Adobe Systems Inc., Mountain View CA). Images shown are representative of five to seven separate immunolabeling experiments, using retinæ from three to five different litters.

Retinal stereology and cell counting

Retinal cell counts were performed on Adobe Photoshop CS processed images using unbiased stereological procedures (Fox et al., 2008; Giddabasappa et al., 2011). For TH-IR and ChAT-IR ACs, cell counts are presented as cells per 500 μ m² of central retina. Three to five fields from five to seven mice from different treatment groups and litters were counted per wholemounted retina. Values from each retina were averaged to obtain one representative value. For GABA-IR and Dab1-IR ACs, cell counts are represented as number of cells per 40 μ m of linear central retina. For GABA-IR and ChAT-IR ACs, cell counts in the INL and ganglion cell layer were obtained. For each treatment group five to seven non-adjacent retinal sections from five to seven mice from different litters were counted using unbiased stereological procedures (Fox et al., 2008; Giddabasappa et al., 2011). Values from each retina were averaged to obtain one representative value.

Western blotting

For total protein detection by SDS-page Western blot, both retinas were removed, cleaned and frozen at –80°C until used, as described (He et al., 2003; Giddabasappa et al., 2011). Briefly, samples were thawed on ice for 10 minutes in lysis buffer, homogenized, centrifuged, and the supernatant was removed to a new tube. Thirty μ L of each sample was used for Bradford protein estimation (Bradford, 1976). Twenty to 25 μ g of protein was loaded onto an 8% SDS denaturing acrylamide gel, run an hour, transferred onto a PVDF membrane, washed, blocked, and incubated in primary antibody solution overnight at 4°C

(Table 1). Membranes were washed, incubated with secondary HRP-conjugated antibody, washed, and imaged with an enhanced chemiluminescence (ECL Plus: Amersham Biosciences, Piscataway NJ). GAPDH was used as the loading control for all gels. Blots were scanned at high-resolution (600 dpi) and analyzed for protein band size and signal intensity. Each band was compared to its corresponding GAPDH loading control. Densitometry measurements were obtained with Adobe Photoshop CS from non-saturated blots and represent four to five retinas from different litters per treatment group.

RNA Isolation

RNA isolation was conducted using the Trizol method, essentially as described (Chadderton et al., 1997; Xiao et al., 2006). Briefly, cleaned neural retinas from each mouse were transferred to an RNase free tube and stored at -80°C . Retinas were homogenized in 500 μL of Trizol and RNA was extracted and precipitated with chloroform and isopropyl alcohol. After DNaseI treatment, the RNA solution was precipitated with ammonium acetate and ethanol, centrifuged, the RNA pellet was resuspended in DEPC water and stored at -20°C . RNA quantity and purity were measured with a Nanodrop spectrophotometer (ThermoFisher Scientific, Wilmington, DE). Four to five mice from different litters per treatment group were used for each sample.

Real Time-quantitative PCR (RT-qPCR)

Total RNA was synthesized into cDNA, as described (Innis et al., 1990). Briefly, 1 μg of total RNA was used for first-strand cDNA synthesis and added to oligo dT and random hexamer primers according to Bio-Rad specifications. All RT-qPCR experiments were run on the Bio-Rad iCycler platform (Bio-Rad Laboratories, Hercules, CA). RT-qPCR primers were designed and validated within certain parameters: GC content 50–60%, melt temperature $55\text{--}65^{\circ}\text{C}$, no secondary structures, no primer dimers or homodimers. Primer quality analysis was done using IDT DNA's Oligo Analyzer (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). All primers designed were intron spanning. Primers were selected from the Roche Applied Science Universal Probe Library and Assay Design Center database. Primers were tested for alternative sites of homology with NCBI's BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) and the UCSC genome browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>). Primer sequences are shown in Table 2. Primers were validated by melt curve analysis for single peak of fluorescence.

All RT-qPCR experiments were performed in triplicate using SYBR green. β -Actin was used as the internal control. The PCR mixture consisted of 12.5 μL of Bio-Rad supermix, 1 μL of cDNA template and 1.5 μL of gene specific forward and reverse primers all combined in a 0.5 mL PCR tube on a 96-well plate. A no template control, water control and air [no sample] control were run for each plate. The PCR cycle conditions were: 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec. A melt curve analysis was run at the end of each plate to ensure proper performance of primer pairs. This was performed starting at 60°C and increasing the temperature 0.5°C per minute to 95°C . Threshold for the cycle threshold (C_t) values was adjusted manually for each plate to coincide with entrance into exponential growth phase of PCR. C_t values were exported to Microsoft Excel for further analysis. The level of gene expression, relative to controls was determined using $\Delta\Delta C_t$ (Yuan et al., 2008). $\Delta\Delta C_t = \Delta C_t$ of gene of interest - ΔC_t of β -actin. To compare GLE versus control, the fold-change of gene expression was determined using $2^{\Delta\Delta C_t}$. Genes with C_t values ≥ 30 indicated a minimal amount of target nucleic acid and therefore were not used for quantitative between group comparisons as results were not considered reliable.

HPLC determination of dopamine and its metabolites

Mice were fully dark-adapted overnight and all procedures were performed under dim red illumination ($\lambda = 650$ nm) as described (Fox et al., 2008; Sriram et al., 2002). Briefly, light- and dark-adapted adult retinas were dissected, cleaned, placed in eppendorf tubes and stored at -80°C until used. Frozen samples were thawed on ice, homogenized in perchloric acid containing dihydroxybenzylamine (DHBA) as internal standard, and centrifuged. The supernatant was filtered through a $0.2\text{-}\mu\text{m}$ membrane, and a $10\text{ }\mu\text{l}$ aliquot was injected from a temperature-controlled (4°C) automatic sample injector connected to a Waters 515 HPLC pump. DA and its metabolites, DOPAC and HVA, were quantified by HPLC. Recovery of each analyte was adjusted with the internal standard and quantified from a standard curve. Values were calculated as pg/retina and represent three to five mice from different litters per treatment group. The DOPAC/DA and HVA/DA concentration ratios estimate DA release and/or turnover (Boireau et al., 1990).

Statistical analyses

For a given set of data, only one animal per litter was used and values represent four to seven animals per treatment group. Data were analyzed by an ANOVA followed by post-hoc multiple comparisons using Tukey's Honestly Significant Difference test. Data are presented as the mean $\pm$ SEM. In graphs and tables, values with $p < 0.05$ or $p < 0.01$ were considered significantly different from controls and were noted where appropriate by single or double asterisks, respectively. In the text, values noted as significantly different from controls had $p < 0.05$. Graphs were generated with KaleidaGraph 4.0 (Synergy Software, Reading PA).

Results

GLE produced a dose- and region-dependent decrease in the number of DA ACs

In controls, the number of TH-IR DA ACs was not significantly different between dorsal and ventral retina (Figure 1; Table 3). GLE produced a significant dose- and region-dependent decrease in the number of TH-IR DA ACs (Figure 1; Table 3). In dorsal retina, LD, MD and HD GLE decreased the mean number of DA ACs by 33%, 43% and 56%, respectively, compared to controls. In ventral retina, LD, MD and HD GLE decreased the mean number of DA ACs by 9%, 27% and 39%, respectively, compared to controls (Table 3). At each GLE dose, the number of DA ACs in the dorsal retina was decreased significantly relative to the ventral retina (Table 3).

To confirm the loss of DA ACs, three additional complimentary experiments were performed using the MD GLE group. First, wholemounts were co-labeled with anti-TH and anti-DDC antibodies: the latter enzyme converts L-DOPA to DA (Nguyen-Legros et al., 1994; Proll et al., 1982). In control and GLE retinas, all TH-IR DA ACs were DDC-IR (Figure 2A). In GLE dorsal retinas, the number of DDC-IR cells significantly decreased by $41.1 \pm 3.9\%$ compared to controls (Figure 2A). Second, we used TH::RFP transgenic mice (Zhang et al., 2004) and co-labeled the wholemounts with anti-TH and TH::RFP-IR antibodies. In control and GLE wholemounts all TH-IR DA ACs were TH::RFP-IR (Figure 2B), demonstrating that these are Type 1 DA ACs (Zhang et al., 2004). In GLE dorsal retinas, the number of TH::RFP-IR DA ACs significantly decreased by $43.8 \pm 5.1\%$ compared to controls (Figure 2B). The GLE-induced decrease in density of the dopaminergic S1 processes is evident in Figures 2A and 2B. Third, Western blots showed that TH significantly decreased by $21.9 \pm 2.4\%$, $37.1 \pm 4.1\%$ and $53.0 \pm 5.9\%$ in LD, MD and HD GLE retinas, respectively, compared to controls (Figure 3). In controls, the Ct value for TH gene expression, using two different primers, was ≥ 30 . Therefore, we could not reliably examine the relative TH gene expression in GLE mice.

GLE decreased the dopaminergic S1 plexus immunoreactivity

In GLE retinas, compared to controls, the immunostaining and number of DA AC processes located in the S1 lamina of the IPL (Nguyen-Legros et al., 1997; Versaux-Botteri et al., 1984; Voigt and Wassle, 1987) were dose-dependently decreased (Figure 2; Table 1). The decreased density of the S1 DA AC processes in MD GLE retinas is evident in Figures 2A and 2B. Fixed-frozen vertical sections show a TH-IR DA AC and its processes in the S1 lamina (Figure 2C), as described (Haverkamp and Wassle, 2000). Calretinin is a calcium-binding protein that immunolabels ACs in the INL, displaced ACs in the ganglion cell layer, RGCs, and S2, S3 and S4 laminae in the IPL (Haverkamp and Wassle, 2000). The distal and proximal strata are from ChAT-IR ACs (Haverkamp and Wassle, 2000). Calretinin-IR, which was not different in GLE retinas (Figure 2C), was used to characterize the distal INL and IPL strata. TH-IR was decreased in the IPL S1 of GLE retinas (Figure 2C).

GLE did not affect the number of ChAT-IR or Dab1-IR glycinergic All ACs

Figure 2C shows that the location and size of the ChAT-IR strata in the IPL of control and GLE retinas are similar. To determine the number of ChAT-IR ACs in the INL, wholemount and fixed-frozen vertical section experiments were conducted. As seen in the dorsal retinal wholemount images in Figure 4, the number of ChAT-IR ACs were not different between treatment groups. Table 3 shows that the number of ChAT-IR ACs was not significantly different in dorsal or ventral retina of control and GLE mice and that there was no significant effect of GLE on the number of ChAT-IR cells on vertical sections. Consistent with these results, Western blots show that the ChAT protein content was not significantly different in GLE retinas compared to controls (Figure 3). Furthermore, the number of Dab1-IR glycinergic ACs (Rice and Curran, 2000), which are postsynaptic to DA ACs (Voigt and Wassle, 1987), and GABAergic ACs in the INL were not significantly different in GLE retinas (Table 3).

GLE did not affect MAO-A retinal protein or gene expression

First, the location and expression of MAO-A in control mouse retinas were characterized, since this had not been described in any species and MAO-A is the predominant MAO isozyme in mammalian retina (Fernandez de Arriba et al., 1990; Ghai et al., 2009; Nakajima et al., 1998). In controls, MAO-A was highly expressed in photoreceptor inner segments, the external limiting membrane, outer plexiform layer, INL, IPL and RGCs (Figure 5: top left panel). Double-label confocal microscopy studies show that MAO-A colocalized with the mitochondrial marker COX IV (Figure 5; Johnson et al., 2007). The location and expression level of MAO-A in GLE retinas was not changed compared to controls (Figure 5: lower left panel). Consistent with this finding, the gene expression of *Maoa*, determined with RT-qPCR, was not significantly different in GLE retinas relative to controls (0.92 ± 0.06).

GLE decreased dopamine and dopamine metabolite content in dark- and light-adapted retinas

Retinal DA, DOPAC and HVA content were determined by HPLC. Figure 6A shows that GLE produced a significant dose-dependent decrease in the light-adapted DA content (17–40%) and significantly decreased the light-adapted DOPAC content (43–51%) and HVA content (48–58%). In light-adapted animals, GLE significantly decreased the [DOPAC]/[DA] ratio by 19–31% and the LD and MD exposures have the largest effects (Figure 6B). Similarly, LD and MD GLE significantly decreased the [HVA]/[DA] ratio by 38–39% in light-adapted retinas (Figure 6B). To determine if the light-stimulated TH activity was altered by GLE, DA and DOPAC were measured in dark- and light-adapted control and MD GLE retinas. In controls, as published (Iuvone et al., 1978), the retinal DA content was not significantly different in dark- and light-adapted retinas (Figure 6C). In GLE retinas, the DA

content was decreased significantly by 32–35% during both dark and light adaptation (Figure 6C). In control and GLE retinas, the retinal DOPAC content significantly increased by 222% and 212%, respectively, from the dark-to light-adapted state (Figure 6C). However, in GLE retinas the retinal DOPAC content was decreased significantly by 47–49% in both adaptation states compared to controls (Figure 6C).

Discussion

There were four main interrelated findings in this study. First, confocal and stereological studies showed that GLE selectively and dose-dependently decreased the number of TH-IR and DDC-IR DA ACs and density of their processes in S1 sublamina of the IPL without affecting other AC types. The loss of DA ACs was greater in the dorsal than ventral retina. Second, as shown by immunoblots, GLE dose-dependently decreased the amount of retinal TH protein. Third, confocal and RT-qPCR results showed that GLE did not affect the DA catabolizing enzyme MAO-A. Fourth, HPLC analysis revealed that GLE significantly decreased DA synthesis and utilization/release in dark- and light-adapted retinas.

The major finding was that GLE selectively and dose-dependently decreased the number of early-born TH-IR DA ACs, the density of their process and retinal TH content. These retinal results are surprising for two reasons. First, GLE did not alter the number or relative ratios of other early-born ACs such as GABAergic (GABA-IR), cholinergic (ChAT-IR) or glycinergic (Dab1-IR). These three AC types comprise the majority of retinal ACs, although the plexus of DA ACs covers the entire retina (Jeon et al. 1998; Rice and Curran 2000; Strettoi and Volpini 2002; Witkovsky, 2004). Second, as reported, the number of late-born rods and bipolar cells, but not Müller glial cells, significantly increased in GLE mouse retinas (Giddabasappa et al., 2011). Previously it was reported that adult rats with GLE had decreased retinal DA content and supernormal scotopic ERGs (Fox et al., 2008). Moreover, monkeys with GLE followed by lifetime lead exposure had supernormal scotopic ERGs and DA AC loss, however, other retinal cell types and changes were not examined (Kohler et al., 1997; Lilienthal et al., 1988, 1994). The possibility that the selective loss of DA ACs produces supernormal scotopic ERGs in adult GLE mice is currently under investigation.

The retinal dopaminergic system appears particularly vulnerable to neurotoxic chemicals and developmental insults (Jones and Miller, 2008). For example, the dopaminergic neurotoxin 6-hydroxydopamine selectively kills DA ACs and produces supernormal scotopic ERGs (Oliver et al., 1986; Skrandies and Wässle, 1988) similar to those observed in children, monkeys and rats with GLE (Fox et al., 2008; Lilienthal et al., 1988, 1994; Nagpal and Brodie, 2009; Rothenberg et al., 2002). Furthermore, placental insufficiency reduces the number of TH-IR DA ACs and retinal DA content, but does not affect other retinal ACs (Loeliger et al., 2004). This does not underlie the current selective loss of retinal DA ACs, as there were no significant GLE effects on any dam or pup/mouse measure during gestation or development (Leasure et al., 2008).

The molecular mechanism underlying the GLE-induced decrease in DA ACs is unknown. One possibility is that GLE decreased a trophic factor required for the maintenance and survival of DA ACs. Brain-derived neurotrophic factor (BDNF) is a likely candidate since mammalian retinal DA ACs express the BDNF receptor TrkB (Cellerino and Kohler, 1997), BDNF null mice have a decreased number of TH-IR DA ACs and density of S1 processes (Cellerino et al., 1998), and intraocular BDNF increased the number of TH-IR DA ACs and S1 processes (Cellerino et al., 1998). BDNF is a downstream target for Nurr1, the gene responsible for transactivation of TH (Volpicelli et al., 2007). Consistent with this idea, a recent report showed that embryonic hippocampal neurons cultured for seven days in one micromolar lead had decreased expression of proBDNF and release of BDNF (Neal et al.,

2010). Alternatively, during GLE the birth of DA ACs may decrease and/or programmed apoptotic cell death may increase: possibilities that are currently being addressed.

The second major finding is that GLE dose-dependently decreased the light-adapted retinal DA content and decreased the major DA metabolites DOPAC and HVA. Moderate-level GLE also decreased the dark-adapted retinal DA and DOPAC content. These results are similar to those in rats with an identical GLE protocol (Fox et al., 2008). The dose-dependent and light-dependent decreases in DA content are internally consistent with the loss of DA ACs and retinal TH content. The finding that the relative dark- and light-adapted DA levels were maintained in controls and GLE retinas indicates that the TH enzyme in the remaining DA ACs functioned normally (Iuvone et al., 1978). The decreased retinal DOPAC and HVA levels as well as the [DA]/[DOPAC] and [DA]/[HVA] ratios in GLE mice indicate decreased DA utilization and release (Boireau et al., 1990). This likely does not result from alterations in DA catabolism since there were no GLE-induced changes in MAO-A protein or gene expression. GABAergic ACs, which are presynaptic to DA ACs, inhibit DA release through GABA-A receptors on the postsynaptic cell surface (Contini and Raviola, 2003; Gustincich et al., 1997; Gustincich et al., 1999). This suggests that the GABAergic system may be altered in GLE retinas: a possibility under examination.

For three reasons, it appears unlikely that the changes in retinal dopaminergic release and utilization in PN60 GLE mice resulted from a direct presynaptic effect of lead. First, the peak retinal [Pb] in LD and HD GLE mice on PN10 was equivalent to 0.4 and 1.2 μM , respectively, (Leasure et al., 2008). Second, the blood and retinal [Pb] were not different from controls after PN30 (Leasure et al., 2008). Third, high micromolar concentrations of lead are required to inhibit rat brain TH activity, depolarization- or spontaneous evoked dopamine release and DA uptake (Jadhav and Ramesh 1997; Minnema et al., 1986; Ramsay et al., 1980). Thus, the mechanisms underlying these alterations remain to be determined.

In summary, the loss and/or dysfunction of DA ACs could produce both functional and structural alterations. For example, a GLE-induced loss of DA ACs during early development might contribute to the increased number of rods and bipolar cells (Giddabasappa et al., 2011) since DA helps regulate neuronal proliferation and development in the retina and brain (Ilia and Jeffery, 1999; Ohtani et al., 2003; Todd 1992). In addition, the loss of retinal DA cells might lower the tolerance to insult by other factors (Frank-Cannon et al., 2008), which could trigger retinal spatial tuning alterations seen in Parkinson's disease (Peppe et al., 1998). Finally, it remains to be determined whether the loss of DA ACs and decreased DA release alters circadian rhythms, alters dark and light adaptation, and/or decreases spatial contrast sensitivity (Jacoby et al., 2011; Kothmann et al., 2009; Ruan et al., 2008; Witkovsky, 2004).

Highlights

- Peak [BPb] in control, low-, moderate- and high-dose newborn mice with gestational lead exposure: ≤ 1 , ≤ 10 , 25 and 40 $\mu\text{g/dL}$
- Gestational lead exposure dose-dependently decreased the number of TH-immunoreactive dopaminergic amacrine cells
- Gestational lead exposure selectively decreased dopaminergic, but not GABAergic, glycinergic or cholinergic, amacrine cells
- Gestational lead exposure dose-dependently decreased retinal dopamine content, its metabolites and dopamine utilization

- A decrease in dopamine can alter ERG amplitudes, circadian rhythms, dark/light adaptation and spatial contrast sensitivity

Abbreviations

AC	amacrine cells
[BPb]	blood lead concentration
ChAT	choline acetyltransferase
COX IV	cytochrome oxidase IV
DA	dopamine
Dab1	disabled 1
DDC	dopa decarboxylase
DOPAC	3,4-dihydroxyphenylacetic acid
ERG	electroretinogram
HD	high dose
HPLC	high-pressure liquid chromatography
HVA	homovanillic acid
INL	inner nuclear layer
IPL	inner plexiform layer
IR	immunoreactive
GLE	gestational lead exposure
LD	low dose
MAO-A	monoamine oxidase-A
MD	moderate dose
PN	postnatal day
RGC	retinal ganglion cells
S1	sublamina 1
TH	tyrosine hydroxylase

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Figure 1. Gestational lead exposure (GLE) produced a dose- and region-dependent decrease in the number of tyrosine hydroxylase immunoreactive (TH-IR) dopaminergic amacrine cells (DA AC)

Confocal images of control, low- (LD), moderate- (MD) and high-dose (HD) GLE wholemount retinas show a significant dose-dependent decrease in the number of TH-IR DA ACs. The images are from dorsal retina, which had a greater loss of DA ACs than the ventral retina (Table 3). Images are representative of four to seven different retinas per treatment group. Scale bar = 80 μ M.

Figure 2. Moderate-dose GLE decreased the number of DOPA decarboxylase (DDC)-IR and TH-RFP-IR DA ACs and their associated inner plexiform layer (IPL) sublamina 1 (S1) plexus (A–B) Confocal images of the dorsal region of retinal wholemounts show that MD GLE significantly decreased the number of (A) DDC-IR and TH-IR co-labeled DA ACs and (B) TH::RFP-IR and TH-IR co-labeled DA ACs. All TH-IR DA ACs are either DDC-IR or TH::RFP-IR. The number and density of TH-IR S1 processes was decreased in GLE retinas. Images are representative of four to seven different retinas per treatment group. Scale bar = 80 μ M. (C) Confocal images from fixed-frozen vertical sections show that TH-IR processes in the S1 sublamina (arrows) are markedly decreased in MD GLE retinas compared to controls. Double-label confocal experiments with calretinin, which labels ACs, retinal

ganglion cells (RGCs) and IPL sublaminae S2, S3 and S4, reveal no co-labeling with TH and no significant differences between treatment groups. Images are representative of four to five different retinas per treatment group. Scale bar = 20 μm .

Figure 3. Western blots show that GLE dose-dependently decreased retinal TH expression
 LS, MD and HD GLE significantly decreased TH expression by $21.9 \pm 2.4\%$, $37.1 \pm 4.1\%$ and $53.0 \pm 5.9\%$, respectively. The expression of choline acetyltransferase (ChAT), that co-labels with calretinin in IPL S2 and S4 (Haverkamp and Wässle, 2000), showed no significant treatment-related differences. GAPDH was used as a loading control. Representative Western blots are from four to five retinas from four to five different litters per treatment group. Values represent the mean $\pm$ SEM and were considered significant from controls if $p < 0.05$.

Figure 4. GLE did not change the number of inner nuclear layer (INL) choline acetyltransferase (ChAT-IR) ACs

Confocal images of the dorsal region of retinal wholemounts from control, LD, MD and HD GLE mice reveal that the number of ChAT-IR ACs in the INL was not different between treatment groups. Similar results were seen in the ventral region and with fixed-frozen vertical sections (Table 3). Images are representative of four to seven different retinas per treatment group. Scale bar = 40 μ M.

Figure 5. GLE did not change the pattern or labeling intensity of monoamine oxidase A (MAO-A)

Fixed-frozen vertical sections show that MAO-A intensely labeled the entire retina, except for the outer nuclear layer (ONL). Since no previous retinal MAO-A confocal studies are published, double-label confocal experiments characterized its colocalization with the mitochondrial marker, COX IV. MAO-A and COX IV are colocalized and highly expressed in the inner segments (IS) and outer plexiform layer (OPL), while they are colocalized and moderately expressed in the IPL. In the INL, MAO-A-IR was greater than that of COX IV-IR. The location and expression level of MAO-A was not changed by MD GLE. Images are representative of four to five different retinas per treatment group. Scale bar = 20 μm .

Figure 6. GLE decreased DA metabolism in light- and dark-adapted retinas (A–C) * $p < 0.05$ and ** $p < 0.01$ compared to corresponding controls. Values that share the same superscript are significantly different at $p < 0.05$. Retinal DA, DOPAC and HVA content were determined by HPLC. (A) In light-adapted retinas, GLE significantly and dose-dependently decreased retinal DA, DOPAC and HVA content. (B) In light-adapted retinas, the [DOPAC]/[DA] and [HVA]/[DA] ratios were decreased significantly in retinas from GLE mice. (C) In controls, the retinal DA content was similar in dark- and light-adapted retinas and the retinal DOPAC content increased during light adaptation. In MD GLE retinas, the retinal DA content was decreased significantly relative to controls, however, it was similar in dark- and light-adapted retinas. Similarly, in MD GLE retinas, the retinal

DOPAC content was decreased significantly, but it increased during light adaptation. [DOPAC]/[DA] was decreased significantly for each GLE treatment compared to control, while [HVA]/[DA] was decreased significantly in LD and MD GLE, but not in HD GLE compared to controls. In light-adapted retinas [DA] was unchanged in MD GLE mice, while [DOPAC] was decreased significantly compared to controls.

Table 1

Primary antibodies used for immunolabeling experiments.

Primary Antigen	Immunogen	Structure Labeled	Host	Source	Dilution	Reference
Tyrosine Hydroxylase (TH)	Native tyrosine hydroxylase from rat pheochromocytoma	Dopaminergic Amacrine Cells	Sheep	Chemicon, AB1542	1:500	Haycock and Waymire, 1982
DOPA Decarboxylase	Human, full length recombinant	Dopaminergic Amacrine Cells	Rabbit	ProteinTech, 10166-1-AP	1:500	Ming, 2009
DsRed	C-terminus of DsRed of <i>Discozoma</i> sp.	RFP protein	Goat	Santa Cruz, sc-33353	1:250	Wall et al., 2000
Choline Acetyl-transferase (ChAT)	Human placental enzyme	Cholinergic Amacrine Cells	Rabbit	Chemicon, AB143	1:500	Reiner and Vincent, 1987
Calretinin	Rat, full length recombinant	Amacrine Cells	Rabbit	Chemicon, AB5054	1:1000	Lee et al., 2006
Monoamine oxidase A (MAO-A)	Human, amino acids 458–527	Monoamine Oxidase A	Rabbit	Santa Cruz, 20156	1:100	Wyatt et al., 1973

Table 2

Gene-specific forward and reverse RT-qPCR primers for genes associated with the dopaminergic system.

Gene Name	Tyrosine Hydroxylase (Th)	Monoamine oxidase A (Maoa)
Forward Primer 5' – 3'	CCCAAGGGCTTCAGAAGAG	AGGTGGCTCTGGCCAAATAAGTGA
Reverse Primer 5' – 3'	GGGCATCCTCGATGAGACT	ACCGGTGGGATGGCACTAATTACA
Accession Number	NM_009377	NM_173740
Amplicon Length (Base Pairs)	61	65
Product Melting Temperature (°C)	82.5	87.5

Primers were designed with either Roche primer database or Integrated DNA Technologies (IDT) Primer QuestSM. Oligo primers were analyzed with IDT Oligo Analyzer 3.1 for melting temperature, GC content and secondary structure.

Table 3

Quantification of amacrine cell populations in the inner nuclear layer (INL)

	Cell Counts	Control	Low Lead	Moderate Lead	High Lead
Wholem mounts ^a	TH (Dorsal)	16.5 ± 0.3	11.1 ± 0.7*	9.4 ± 0.5**	7.3 ± 0.6**
	TH (Ventral)	17.3 ± 0.6	15.8 ± 0.3*	12.7 ± 0.4*	10.5 ± 0.3**
	ChAT (Dorsal)	367 ± 16	371 ± 19	375 ± 21	359 ± 24
	ChAT (Ventral)	372 ± 19	368 ± 22	368 ± 25	363 ± 18
Vertical Sections ^b	ChAT	16 ± 3	17 ± 4	17 ± 3	16 ± 4
	Dab1	17 ± 3	16 ± 4	15 ± 5	17 ± 4
	GABA	7 ± 1	8 ± 1	7 ± 1	8 ± 2

TH: Tyrosine hydroxylase; IR: Immunoreactive; ChAT: Choline acetyltransferase; Dab1: Disabled 1

^a Values represent the mean ± SEM for 500 μm² of dorsal or ventral retina from three to nine different whole mounted mouse retinas per treatment group.^b Values represent the means ± SEM for 40 μm fixed-frozen transverse sections from three non-adjacent slides for three to five different mouse retinas per treatment group.

* p < 0.05 and

** p < 0.01 compared with corresponding controls.