Low-Level Gestational Lead Exposure Increases Retinal Progenitor Cell Proliferation and Rod Photoreceptor and Bipolar Cell Neurogenesis in Mice

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Perinatal exposure to environmental toxicants such as lead, methylmercury, pesticides, and polychlorinated biphenyls increases the risk of developmental disabilities, mental retardation, neurosensory alterations, and psychiatric morbidity (Grandjean and Landrigan 2006; Mendola et al. 2002). The spectrum of neurotoxic effects depends upon the perinatal period of exposure, concentration and duration of exposure, and genetic susceptibility (Grandjean and Landrigan 2006; Rice and Barone 2000). This is exemplified best by lead exposure, which is characterized by the number of neurons in the rod signaling pathway and to determine the cellular mechanisms underlying the phenotype.

RESULTS: Blood lead concentrations ([BPb]) in controls and after low-, moderate-, and high-dose GLE were ≤ 10 µg/dL—approximately 25%, and approximately 40 µg/dL, respectively, at the end of exposure (postnatal day 10 [PND10]); by PND30 all [BPb] measures were ≤ 1 µg/dL. In vivo BrdU (5-bromo-2-deoxyuridine) pulse-labeling and Ki67 labeling of isolated cells from developing mice showed that GLE increased and prolonged retinal progenitor cell proliferation. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) and confocal studies revealed that the rod photoreceptors and rod and cone bipolar cells (BCs), but not Müller glial cells, increased in a nonmonotonic manner by 16–30% in PND60 GLE offspring. Retinal lamination and the rod/cone BC ratio were not altered. In vivo BrdU (5-bromo-2-deoxyuridine) pulse-labeling and Ki67 labeling of isolated cells from developing mice showed that GLE increased and prolonged retinal progenitor cell proliferation. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) and confocal studies revealed that GLE did not alter developmental apoptosis or produce retinal injury. BrdU birth-dating and confocal studies confirmed the selective rod and BC increases and showed that the patterns of neurogenesis and gliogenesis were unaltered by GLE.

CONCLUSIONS: Our findings suggest two spatiotemporal components mediated by dysregulation of different extrinsic/intrinsic factors: increased and prolonged cell proliferation and increased neuronal (but not glial) cell fate. These findings have relevance for neurotoxicology, pediatrics, public health, risk assessment, and retinal cell biology because they occurred at clinically relevant [BPb] and correspond with the ERG phenotype.

KEY WORDS: bipolar cells, development, gestational exposure, glia, lead, mice, neurogenesis, proliferation, retina, rod photoreceptors.

Materials and Methods

Animal model. All experimental and animal care procedures complied with the National Institutes of Health (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 regard for alleviation of suffering. The GLE model, as described by Leasure et al. (2008), uses C57BL/6 mice. Briefly, female mice fed...
Purina lab chow 5001 (Purina Mills, St. Louis, MO) were given water (control) or water containing a low (27 ppm), moderate (55 ppm), or high (109 ppm) concentration of lead 2 weeks before mating, during pregnancy, and through PND10 to produce a human-equivalent GLE. Dams were mated with a control male overnight, and the presence of a vaginal plug was recorded as gestation day (GD) 0.5. On PND10, we replaced the lead solutions with water. Litter sizes were maintained at six pups each until weaning (PND21). Mice were sacrificed by decapitation between 1000 and 1200 hours on PND1, PND3, PND5, PND7, PND10, and PND60. We observed no differences between sexes for any end point.

As described by Leasure et al. (2008), control, LD, MD, and high-dose (HD) GLE groups had peak [BPb] on PND0 and/or PND10 of 0.72 ± 0.07, 10.10 ± 0.65, 27.23 ± 1.39, and 42.06 ± 0.70 µg/dL, respectively; on GD14 the dam’s [BPb] was similar to that of PND0 pups; and on PND30 the [BPb] in GLE mice was not different from those in controls (n = 10–15 mice/treatment group/age). There were no statistical differences between control and GLE groups on any dam measure, litter measure, or body weight.

**Retinal epithelium, light, and immunocytochemistry/confocal microscopy studies.** All tissue processing, image acquisition, and analysis procedures were as described previously (Fox et al. 1997, 2008; He et al. 2003; Johnson et al. 2007). Briefly, mice were removed and immersed in ice-cold phosphate-buffered saline (PBS), corneas were slit, and eyes were immersion fixed in buffered 4% paraformaldehyde for 30 min (confocal microscopy) or Karnovsky’s fixative (light microscopy). For confocal microscopy, central sections (10 µm thick) from cryoprotected frozen retinas were cut at 200–400 µm from the optic nerve. Nuclear dyes and primary antibodies directed against retinal-cell-type-specific and cell-cycle markers were used (Table 1). ONL and INL thickness was measured on 4’.6-diamidino-2-phenylindole (DAPI)-stained slides using a calibrated micrometer eyepiece. For light microscopy, superior central retinal sections were embedded in Araldite (Electron Microscopy Sciences, Hatfield, PA), sectioned (1 µm), and stained with toluidine blue for rod and cone photoreceptor counting. Twenty fields, each 100 µm in length, were examined.

For all studies, studies (three nonadjacent coded sections) from each treatment group were analyzed using identical exposure and scanning parameters. The images were processed identically using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). All cell counts were conducted using Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD) by an observer who was masked to the exposure type and who used unbiased stereology and morphologic criteria, as described previously (He et al. 2003; Lucocq 2007).

For pulse-labeling cells in S phase (Rachle et al. 2002), BrdU (50 µg/g) was injected intraperitoneally (IP) into pregnant dams on GD16.5 and GD18.5, and pups were injected on PND1, PND3, PND5, and PND7. Mice were sacrificed 2.5 hr after injection. For birth dating late-born retinal cells, two doses of BrdU (100 µg/g) were injected into PND1, PND3, PND5, and PND7 pups separated by 2 hr, and mice were sacrificed on PND60. Slides were triple labeled with an anti-BrdU antibody, the nuclear stain DRAQ5, and antibodies for rods, BCs, or MGCs and then processed for confocal microscopy and counting as previously described (He et al. 2003; Johnson et al. 2007). We used staining, morphological, and pixel density criteria (≥ 20 pixels at 300 dpi) to identify and count BrdU-immunoreactive (BrdU-IR) cells, as previously described (Rachel et al. 2002).

**Western blotting.** Immunoblotting was performed as described previously (Fox et al. 2008; He et al. 2003). Briefly, both retinas were removed, cleaned in PBS, and frozen at –80°C until use. Thawed retinas were homogenized in lysis buffer and centrifuged, and 20–30 µg protein was loaded onto SDS-PAGE gels. Blots were probed with selected primary antibodies using GADPH as a loading control (Table 1), incubated with horseradish peroxidase–conjugated secondary antibody, and visualized with ECL Plus (GE Healthcare, Piscataway, NJ). Densitometry measurements were obtained using ImageJ software (NIH 2009) from nonsaturated blots.

**Immunolabeling dissociated progenitor cells.** We followed the procedure for single-cell dissociation of PND2, PND4, and PND6 retinas as described previously by He et al. (2000), except that we replaced HEPES with Hank’s balanced salt solution. Dissociated cells (10^6 cells/mL) were pipetted onto slides, fixed with 4% paraformaldehyde, rinsed, and air dried. Coded slides were double labeled for Ki67 and DRAQ5 and processed for confocal microscopy and counting as previously described (He et al. 2000).

**Terminal deoxynucleotidyl transferase DUTP nick end labeling (TUNEL).** Apoptotic cells were labeled using the ApoAlert DNA Fragmentation Assay TUNEL Kit (Clontech, Mountainview, CA) according to manufacturer’s procedure. Briefly, retinal sections were dried and fixed with 4% paraformaldehyde, incubated with proteinase K, washed, fixed, and washed again. Slides were incubated with equilibrium buffer, sequentially incubated with terminal deoxynucleotidyl transferase (TdT) and sodium chloride/sodium citrate, washed, and coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories). Positive-control (DNase1) and negative-control (no TdT) slides were processed simultaneously for each experiment. Fluorescent apoptotic cells were identified in different retinal layers using stringent morphologic criteria and counted as previously described (He et al. 2000, 2003).

**Statistical analysis.** Only one animal per litter was used for each measure. Data

| Table 1. Cell-specific primary antibodies and dyes. |
|---------------------------------|------------|---------------------------------|------------------|
| Name                           | Structure labeled or targeted | Host                     | Product number and source                          | Dilution |
| BrdU                            | S-phase marker                  | Rat                      | Ab6326, Abcam Inc., Cambridge, MA                | 1:40     |
| ChAT                            | Amacrine cells                  | Rabbit                   | Ab143, Chemicon, Temecula, CA                   | 1:100    |
| Chx10                           | BCs                              | Sheep                    | X1180P, Exalpa Biologicals Inc., Shirley, MA     | 1:50     |
| Cyclic D3                       | MGC nucleus                     | Mouse                    | MCA1886; Serotec, Raleigh, NC                    | 1:40     |
| DAPI                            | Nucleus                          | H-1200, Vector Laboratories, Burlingame, CA | 1.5 µg/mL |
| DRAQ5                           | Nucleus                          | BOS-899-001; Alexis Biochemicals; Enzo Life Sciences International Inc., Plymouth Meeting, PA | 20 µM |
| Glutamine synthetase            | MGCs                             | Rabbit                   | G2781, Sigma Aldrich, St. Louis, MO              | 1:5,000  |
| Glyceraldehyde 3-phosphate dehydrogenase | Protein loading control     | Mouse                    | MAB 374, Millipore, Billerica, MA                | 1:300    |
| Kit87                           | Progenitor cell marker           | Mouse                    | 500069; BD, Franklin Lakes, NJ                   | 1:100    |
| Opsins (middle- and short-wavelength sensitive) | Cones                 | Rabbit                   | Gifts from C. Craft, University of Southern California, Los Angeles, CA | 1:1,000  |
| PH3                             | M-phase marker                   | Rabbit                   | 56-0701; Upstate Biotech, Lake Placid, NY        | 1:40     |
| PKCα                            | Rod BCs                          | Rabbit                   | P4334, Sigma Aldrich                             | 1:10,000 |
| Rhodopsin                       | Rod photoreceptors               | Mouse                    | MAB5356, Chemicon                               | 1:5,000  |
represent four to seven retinas, each from a different mouse, at each age per treatment group. Group data were analyzed by analysis of variance followed by post hoc multiple comparisons using Tukey’s honestly significant difference test (KaleidaGraph; Synergy Software, Reading, PA). Data are presented as mean ± SE, and the difference from controls was regarded as significant if \( p < 0.05 \).

**Results**

**GLE increased retinal ONL and INL thickness.** DAPI nuclear staining revealed that GLE increased the cell density and thickness of the ONL and INL in PND60 mice (Figure 1A).

Figure 1. GLE selectively increases the number of rod photoreceptors and BCs in the adult (PND60) mouse retina as shown in light and confocal microscopy studies. (**A**) Representative DAPI nuclear labeling shows that ONL (rod and cone nuclei), INL (horizontal, bipolar, amacrine, and MGC nuclei), outer plexiform layer (OPL), inner plexiform layer (IPL), and total retinal thickness increased in retinas from animals in the LD, MD, and HD GLE groups. Retinal GCL cellularity and retinal lamination were not different in control and GLE retinas. (**B–E**) Representative double-labeled confocal microscopy studies reveal rod and BC selectivity of the retinal phenotype. (**B**) The number of rhodopsin-IR rod nuclei and PKC\(\alpha\)-IR rod BC somas—in distal (upper) INL—increased in GLE retinas. (**C**) The numbers of cone outer segments (OS) immunoreactive for middle- and short-wavelength-sensitive opsin (M-/S-opsin) and horizontal cells immunoreactive for calbindin were not different in control and GLE retinas. (**D**) The numbers of Chx10-IR BC nuclei and Chx10/PKC\(\alpha\) colabeled rod BCs increased in adult GLE retinas. (**E**) The number of cyclin D3–IR MGCs colabeled with glutamine synthetase (GS), were similar in adult control and GLE retinas. Bars = 20 µm for **A** and for **B–E**.
GLE also increased the thickness of the outer and inner plexiform (synaptic) layers and total retinal thickness (Figure 1A). The central ONL and INL of controls contained 10–11 and 4–5 nuclei/layer, respectively, and were 54.0 ± 2.4 and 37.2 ± 2.1 µm thick (mean ± SE), respectively. In LD, MD, and HD GLE mice, ONL thickness significantly increased by 18.3 ± 1.5%, 25.5 ± 2.2%, and 8.4 ± 1.2%, respectively; INL thickness significantly increased by 21.4 ± 1.6%, 29.8 ± 2.5%, and 18.0 ± 1.9%, respectively; and total retinal thickness significantly increased by 25.7 ± 2.1%, 30.4 ± 2.8%, and 15.7 ± 1.5%, respectively. The number of DAPI-stained nuclei in the retinal ganglion cell layer (GCL) of controls (15.4 ± 1.5 per 100 µm length) was not significantly different in GLE mice (Figures 1A and 2A).

**GLE selectively increased the number of rods.** We conducted additional studies to characterize and quantify the cell-specific increased in ONL thickness. Rhodopsin-labeled retinas showed that GLE increased ONL cellularities and thickness (Figure 1B). In GLE mice, the density of cone outer segments immunoreactive for middle- and short-wavelength-sensitive opsins was not significantly different from controls (Figure 1C; 15.4 ± 0.8 per 100 µm length). Stereologic analysis showed that GLE selectively and significantly increased the number of rods (Figure 2A). In controls, the numbers of central rod and cone nuclei were 126.5 ± 3.4 and 3.9 ± 0.2 per 100 µm length, respectively, consistent with previously published results (He et al. 2003). In LD, MD, and HD GLE mice, the numbers of rods significantly increased by 16.9 ± 4.7%, 27.3 ± 5.3%, and 9.5 ± 1.6%, respectively; however, cone density was unchaged (Figure 2A). Immunoblots showed that the rhodopsin content significantly increased in LD, MD, and HD GLE retinas by 21.1 ± 1.5%, 34.3 ± 2.7%, and 14.0 ± 2.6%, respectively (Figure 2B). Collectively, these findings demonstrate that GLE increased the number of rod, but not cone, photoreceptors in PND60 mice. Furthermore, these nonmonotonic effects for the stereology and Westerns blots were characterized by an inverted U-shaped dose–response curve.

**GLE selectively increased the number of BGs.** Additional studies were conducted to determine the INL cell types that increased. We double-labeled retinal sections with an anti-Chx10 antibody, a selective marker for differentiated rod and cone BCs, and an anti-protein kinase Cα (anti-PKCα) antibody that selectively labels rod BCs (Green et al. 2003). In GLE retinas, the number of Chx10-IR BCs increased (Figure 1D). In controls, we observed 56.8 ± 4.5 Chx10-IR cells per 100 µm length of central retina. In LD, MD, and HD GLE mice, this significantly increased by 16.3 ± 4.7%, 26.7 ± 4.7%, and 19.8 ± 5.2%, respectively (Figure 2A). Immunoblots showed that Chx10 content significantly increased in LD, MD, and HD GLE retinas by 18.3 ± 1.6%, 31.4 ± 2.8%, and 20.4 ± 3.1%, respectively (Figure 2B). In GLE retinas, the number of PKCα-IR rod BCs exhibited similar increases (Figure 1D). The number of PKCα-IR rod BCs in control central retina was 18.4 ± 1.5 per 100 µm length, and they significantly increased in LD, MD, and HD GLE mice by 17.9 ± 3.1%, 24.9 ± 3.7%, and 18.9 ± 2.7%, respectively (Figure 2A). This yielded rod BC:total BC ratios of 0.31–0.34 for control and GLE retinas, which are normal ratios for mammalian retina (Strettoi and Volpini 2002). Immunoblots showed that PKCα content significantly increased in LD, MD, and HD GLE retinas by 19.4 ± 1.4%, 24.8 ± 2.2%, and 21.0 ± 2.5%, respectively (Figure 2B). In control and GLE mice, the density of calbindin-IR horizontal cells (Figure 1C) and cyclin D3-IR MGCs (Figure 1E) in central retina (1.4 ± 0.1 and 10.5 ± 0.5 per 100 µm length, respectively) was not significantly different (Figure 2A). Immunoblots for glutamine synthetase revealed no significant differences (Figure 2B), consistent with the confocal and morphometric MGC results (Figures 1E and 2A). We observed no glial fibrillary acid protein labeling or content change in any retinas (Fox DA, Giddabasappa A, Chaney S, unpublished data), similar to our results in GLE rats (Fox et al. 2008), indicating that GLE produced no injury response. The density of choline acetyltransferase-IR (ChAT-IR), γ-aminobutyric acid–IR, and Disabled-1–IR (Dab1 glycergic AII) amacrine cells in control central INL was 1.9 ± 0.2, 7.3 ± 0.5, and 4.8 ± 0.3 per 100 µm length, respectively. This yielded a Disabled-1:ChAT INL amacrine cell ratio of 2.5:1, which is consistent with our calculated values from published data (Jeon et al. 1998; Rice and Curran 2000). We found no significant differences in these amacrine cell densities in GLE retinas (Figure 2A). Immunoblots for ChAT confirmed the stereology results (Figure 2B). These composite results demonstrate that GLE selectively and proportionately increased the number of rod and cone BCs without altering the cell fate of other cells. Furthermore, the nonmonotonic effects were characterized by an inverted U-shaped dose–response curve.

**BrdU birth-dating studies.** To determine the spatiotemporal kinetics of late-born retinal cells, we conducted BrdU double-labeling experiments with anti-rhodopsin (Figure 3A), anti-Chx10 (Figure 3B), or anti-cyclin D3 antibodies (Figure 3C). GLE selectively and

![Figure 2. GLE selectively increases the number of rod photoreceptors and BCs in retinas from adult mice. (A) Unbiased stereological analyses of major retinal cell types in adult retinas show that LD, MD, and HD GLE produced selective and significant nonmonotonic increases in the number of rods and BCs, relative to controls. Values are means ± SE from three nonadjacent sections per retina from four to seven retinas per treatment group, with each retina from a different mouse. (B) Representative Western blots reveal that GLE increased the retinal content of rhodopsin, Chx10, and PKCα but did not change the amount of glutamine synthetase (GS) or amacrine cell ChAT content. GAPDH was used as the protein loading control. *p < 0.05, compared with control. Groups sharing # or † were significantly different within the cell type at p < 0.05.](image-url)
significantly increased rod and BC neurogenesis by 35–40% from PND1 to PND5 while maintaining normal cellular migration and lamination, which confirms and extends our findings. For control and GLE, the rod, BC, and MGC peak birth dates were PND1, PND3, and PND3, respectively, consistent with previously published results (Young 1985b). Thus, MD GLE did not disrupt the initiation or pattern of neurogenesis or gliogenesis, despite increasing rod and BC neurogenesis by approximately 30% (Figure 2A). Two possible, although not mutually exclusive, mechanisms might underlie these selective changes: increased proliferation and/or decreased apoptosis.

**GLE increased and prolonged the proliferation of RPCs.** We conducted BrdU pulse-labeling studies to determine if the increased rod and BC neurogenesis resulted from increased RPC proliferation. Figure 4A shows that BrdU labeled the central neuroblastic layer (NBL) from GD16.5 to PND3, consistent with the termination of DNA synthesis by PND5 (Young 1985a). The labeling pattern in controls (Figure 4B) was similar to that for [3H]-thymidine labeling in developing rat retinal explants (Alexiades and Cepko 1996). In MD GLE retinas, BrdU labeling persisted until PND5 (Figure 4A,B); we observed no BrdU-IR cells on PND7 (Figure 4B). From GD16.5 to PND3, GLE significantly increased the number of BrdU-IR cells by 30% (Figure 4B).

To assess whether GLE affected the spatiotemporal pattern and/or number of RPCs undergoing mitosis, we double-labeled retinas with anti-phosphohistone H3 (anti-PH3) and anti-BrdU antibodies. PH3 labeled cells only in the apical NBL/subventricular zone, the site of retinal mitosis (Alexiades and Cepko 1996; Barton and Levine 2008; Young 1985a), where approximately 10% colocalized with BrdU cells on GD16.5 and GD18.5 (Figure 4A). Although the spatiotemporal

**Figure 3.** BrdU birth-dating and confocal studies demonstrate that MD GLE selectively and significantly increased the number of rods and BCs in retinas from adult mice. GLE did not alter the kinetics of neurogenesis and gliogenesis. BrdU-IR and rhodopsin-IR rods (A) and BrdU-IR and Chx10-IR BCs (B) increased in GLE retina, compared with controls. (A) No differences in the number of BrdU-IR and cyclin D3–IR MGCs were observed in control and GLE retinas. Values are mean ± SE of IR cells per 400 µm of central retina and represent from four to seven retinas at each age per treatment group, with each retina from a different mouse. *p < 0.05, and **p < 0.01 compared with controls.

**Figure 4.** MD GLE increases and prolongs RPC proliferation in vivo and ex vivo. (A) The M-phase marker PH3 and S-phase marker BrdU are present until PND3 in controls and PND5 in GLE central retina. They colocalize in the distal NBL. At all ages, except PND7, there are more BrdU-IR and PH3-IR cells in the GLE retinas. Abbreviations: inbl, inner NBL; onbl, outer NBL. Bar = 20 µm. Stereological analysis of BrdU (B) and PH3 (C) labeling shows that GLE significantly increased and prolonged RPC proliferation. (D) Dissociated single cells from PND2, PND4, and PND6 control and MD GLE retinas were double labeled with an anti-Ki67 antibody and DRAQ5, results reveal that GLE significantly increased RPCs in an age-dependent manner, similar to that shown in B. In B–D, values are mean ± SE from four to seven retinas at each age per treatment group, with each retina from a different mouse; in B and C, values represent IR cells per 400 µm of central retina. *p < 0.05, and **p < 0.01 compared with control.
pattern of PH3 labeling was similar in control and GLE retinas, GLE significantly increased the number of PH3-IR cells by 25% from GD16.5 to PND3 (Figure 4C).

To explore possible GLE-induced alterations in cell cycle progression, we calculated PH3:BrdU ratios for controls and GLE during the period of significantly increased RPC proliferation. The ratios (0.10–0.12), similar to those obtained with PND1 mouse retina by [3H]-thymidine pulse-labeling (Young 1985), were not significantly different at any age. This indicates that GLE did not alter the relative time spent in S and M phases.

To further confirm the pulse-labeling BrdU proliferation results, we double-labeled dissociated single cells from developing retinas with an anti-Ki67 antibody and the nuclear stain DRAQ5. Figure 4D reveals that 43.6 ± 0.9%, 23.0 ± 1.4%, and 10.5 ± 1.5% of the control retinal cells were proliferating at PND2, PND4, and PND6, respectively, consistent with rodent studies using different techniques to estimate the percentage of proliferating cells (Alexiades and Cepko 1996; Barton and Levine 2008; Young 1985). In GLE retinas, the number of proliferating cells significantly increased on PND2, PND4, and PND6 in GLE by 60.5 ± 1.4%, and 10.5 ± 1.5%, respectively (Figure 4D).

These results, which are consistent with the BrdU data (Figure 4B), demonstrate that GLE increased the proliferation of late-born retinal cells.

**GLE did not alter retinal apoptosis during development.** A GLE-induced decrease in apoptosis could increase the number of proliferating RPCs and differentiated neurons. In the NBL of control and MD GLE retinas, TUNEL-positive cells exponentially and similarly increased from GD16.5 to PND5 (Figure 5A,B). In the GCL of control and MD GLE retinas, TUNEL-positive cells increased linearly from GD16.5 to PND3 and then decreased, resulting in an inverted U-shaped curve (Figure 5C). On PND1 and PND7, TUNEL-positive cells in the GCL were slightly, but significantly, increased and decreased in MD GLE mice, respectively.

However, we found no significant differences in GCL cellularity at PND60 (Figures 1A and 2A). In the ONL of control and GLE retinas, TUNEL-positive cells were low on PND7 and PND10 and not significantly different. Although numbers of TUNEL-positive cells were 20- to 30-times higher in control and GLE INL than in the ONL on PND7 and PND10, there were no significant differences between treatment groups (Figure 5D). The spatiotemporal patterns and amount of apoptosis in controls were similar to previously published results (He et al. 2003; Portera-Cailliau et al. 1994; Young 1984). Thus, the GLE-induced increase in proliferating RPCs and late-born neurons was not due to decreased apoptosis.

**Discussion**

We obtained three novel results with our murine model of GLE. First, GLE produced selective nonmonotonic increases, characterized by an inverted U-shaped dose–response curve, in the numbers of rods and BCs in the adult retina. The increase in rods and BCs did not alter retinal lamination or the proportion of rod and cone BCs. Second, GLE increased and prolonged RPC proliferation without changing the relative S- or M-phase length, initiation of neurogenesis, or pattern of neurogenesis and gliogenesis. Third, GLE did not significantly alter apoptosis or produce reactive gliosis during retinal development and maturation.

The molecular mechanisms responsible for the novel GLE-induced retinal phenotype are unknown. However, the increased and prolonged RPC proliferation accompanied by the selective increase in late-born neurons suggests that two different spatiotemporal components are involved. We deduce that the first involves a modest increase in the number of cell cycles, and the second involves factors that regulate rod and BC fate. Multiple extrinsic and intrinsic factors regulate RPC proliferation and cell fate decisions during development (Livesey and Cepko 2001; Martins and Pearson 2008).

![Figure 5. MD GLE does not change the amount or pattern of retinal apoptosis during development.](image-url)
Because there are no similar GLE studies, we suggest three logical non-mutually exclusive possible mechanisms that could produce the adult GLE phenotype. First, GLE could enhance the progression of the RPC cell cycle mediated by glutamate and AP5 (Matsubara and Ohno 2008). For example, the pharmacological antagonism of glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-D-aspartic acid (NMDA) receptors increases RPC proliferation in developing mouse retinas (Martins and Pearson 2008). The noncompetitive inhibition of receptors for NMDA and kainate observed with 1–5 μM added lead (Alkondon et al. 1990; Musshoff et al. 1995) could increase RPC proliferation. Second, GLE might decrease nitric oxide synthase activity in the developing retina, which would increase RPC proliferation. This idea is consistent with findings that nitric oxide regulates RPC proliferation (Lilienthal et al. 2006) and that moderate-level GLE and lactational lead exposure decreased neuronal nitric oxide synthase in rat brain (Chetty et al. 2001). Third, GLE could selectively increase a subpopulation of RPCs that share a late-born neuronal cell fate, because approximately 20% of all multiple cell clones in rat RPC lineage-tracing studies contained rods and BCs (Turner and Cepko 1987). Thus, this novel GLE model could help uncover the pathways that establish early- and late-born RPCs and specify neuronal versus MGC fate.

Conclusion