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Gestational lead exposure shortens cell cycle length and
activates developmental molecular network of
neurogenesis in postnatal retina**

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1 **Gestational lead exposure shortens cell cycle length and activates developmental**
2 **molecular network of neurogenesis in postnatal retina.**

3
4 **Short Running Title:** Gestational lead exposure shortens cell cycle

5
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23 **ABSTRACT**

24 **Background:** Low level gestational lead exposure (GLE) produces increased number of
25 rod photoreceptors and supernormal electroretinograms (ERGs) in children and rodents.
26 This contrasts with known apoptotic loss of rod photoreceptors and subnormal ERG of
27 high level adult Lead exposure.
28

29 **Objectives:** The goal of this study was to decipher the underlying cellular and molecular
30 mechanisms of this novel GLE phenotype in our murine GLE model. We hypothesized
31 that cell cycle reentry of retinal progenitor cells (RPCs) and cell cycle exit, and rod
32 photoreceptor cell fate specification of RPCs would be higher in GLE relative to control
33 retinas and would occur with concurrent molecular changes.
34

35 **Methods:** Female C57BL/6 mice were exposed to lead through drinking water 2 weeks
36 before mating, throughout gestation till postnatal day 10 (PN10). Blood lead
37 concentrations ([BPb]) in controls and GLE pups were ≤ 1 and 25 $\mu\text{g/dL}$, respectively, at
38 PN10. Retinas from pups aged PN2 to PN10 were used to perform retinal experiments.
39

40 **Results:** Cumulative BrdU labeling revealed that GLE shortens cell cycle length at G1-
41 phase and accelerates cell cycle. Affymetrix gene expression array, qPCR, Western blot
42 and confocal microscopy showed that GLE increases alters NOTCH1-HES1 pathway, cell
43 cycle regulators (Cyclins, INKs, p27KIP, RB phosphorylation) and bHLH (ASCL1, OTX2,
44 HES6) rod photoreceptor differentiation regulators, without change in spatiotemporal
45 expression pattern. In both GLE and control retinas, we found a spatial coupling of
46 regulators of cell cycle exit (p27KIP), cell cycle inhibitors (p16, p19 INKs), cell cycle

47 reentry (HES1), cell fate specification (ASCL1, OTX2, HES1) in the SVZ, while cell cycle
48 progression regulators (Cyclins) were spatially uncoupled and located in the inner retina.
49 ChIP-qPCR showed increased activity of Cyclin D1 and ASCL1 promoters in GLE retinas.

50

51 **Conclusions:** Our findings show that GLE shortens cell cycle to increase proliferation
52 and rod photoreceptor differentiation of RPCs, and this is mediated through differential
53 expression of the NOTCH1-HES1 signaling, cell cycle regulators (Cyclins, INKs, CIP/KIP)
54 and differentiation factors (ASCL1, OTX2). Our results suggest that there is greater cross-
55 talk between the spatiotemporally coupled processes of cell cycle inhibition (INKS, KIP),
56 cell fate specification factors (ASCL1, OTX2, HES1) and cell cycle reentry mediated by
57 HES1 than with spatiotemporally uncoupled cell cycle progression regulation by Cyclins,
58 and this spatiotemporal segregation may provide an insurance to proper transition of
59 RPCs through these developmental stages. These results provide new insights into the
60 molecular mechanism of retinal development and GLE effects.

61

62

63 **Abbreviations:** AC: Amacrine cell, BC: Bipolar cell, BrdU: 5-bromo-2-deoxyuridine, E:
64 embryonic day, GCL: ganglion cell layer, GLE: gestational lead exposure, INL: inner
65 nuclear layer, IR: immunoreactive, LSCM: laser scanning confocal microscopy, NBL:
66 neuroblastic layer, ONL: outer nuclear layer, PH3: phosphohistone H3, PN: postnatal
67 day, RPC: Retinal progenitor cell

68

69

70 **Keywords:** rod photoreceptors, retina, progenitor cells, gestational lead exposure,
71 neurogenesis, cell cycle, cell fate specification, cell cycle exit.

72

73

74 **INTRODUCTION**

75 Although lead continues to be removed from its biggest sources in gasoline, water
76 pipes and paint worldwide, it remains an environmental health concern, especially for
77 children who are most susceptible to lead exposure due to greater absorption of ingested
78 lead and greater vulnerability of their developing nervous system relative to adults
79 (Bellinger 2008; Leggett 1993). The current Centers for Disease Control and Prevention
80 (CDC) level of concern for lead is blood lead concentrations ([BPb]) $< 10 \mu\text{g/dL}$ and there
81 are half a million children between 1 to 5 years old, in U.S. alone with [BPb] $> 5 \mu\text{g/dL}$.
82 Lead is a persistent and potent developmental brain, auditory and retinal neurotoxicant
83 (Bellinger 2008; Laughlin et al. 2009; Mendola et al. 2002; Otto and Fox 1993; Rothenberg
84 et al. 2002) whose pathophysiological effects depend on dosage, duration and age at the
85 time of exposure. For example, lead exposure during postnatal development and at high
86 dosage ([BPb] $> 10 \mu\text{g/dL}$) in monkeys and rodents as well as in occupationally lead-
87 exposed workers or adult rats produces long-term rod photoreceptor-selective visual
88 deficits, decreased rod-mediated (scotopic) electroretinograms (ERGs) and/or rod-
89 selective apoptosis (Fox and Boyes 2013; Fox 2015). In marked contrast, gestational lead
90 exposure (GLE) at low dosage ([BPb] $< 10 \mu\text{g/dL}$) in humans and rodents produces
91 supernormal scotopic ERGs (Fox et al. 2008; Nagpal and Brodie 2009; Rothenberg et al.
92 2002), increased and prolonged proliferation of retinal progenitor cells (RPCs) (Fox et al.
93 2008; Giddabasappa et al. 2011) and increased differentiation but delayed maturation of
94 late-born rods and bipolar cells without changing normal retinal programmed cell death
95 (Chaney et al. 2016; Fox et al. 2008; Giddabasappa et al. 2011). Other studies have
96 shown that low level lead exposure ([BPb] $< 10 \mu\text{g/dL}$) produces deficiency in intellectual
97 ability of children (Canfield et al. 2003; Jusko et al. 2008) and increase susceptibility to
98 late onset neurological diseases, such as Alzheimer's disease (Basha et al. 2005; Wu et
99 al. 2008). Lead exposure produces potent and varied detrimental effects on the brain and
100 visual system of humans and rodent models, where the exact phenotypic outcome is
101 determined by the timing, duration and dose of lead exposure.

102 Several studies have been conducted to determine the underlying molecular
103 mechanism by which lead exerts its adverse effects on the brain and visual system. High
104 lead exposure at 0.4 to 1.9 μM Pb alters global methylation status of neuronal genes
105 (Neurog1, Lhx3, Ptxna4, Efna2 and Grik4 genes in differentiating embryonic stem cell)
106 (Senut et al. 2014) and impairs proliferation through activation of oxidative stress NRF2
107 pathway at 1mM Pb in in vitro neural stem cells (Wagner et al. 2017). Lead induces rod
108 photoreceptor selective apoptosis through calcium overload and cytochrome c-caspase
109 cascade in rodent retinas, and neuronal apoptosis in the brain is thought to also be
110 mediated through calcium binding enzymes (Fox et al. 1997; He et al. 2003; Sobieniecki
111 et al. 2015). Long-term neurological deficits were found due to lead exposure blocking
112 PKC signaling in neurologic tissue (Pokorski et al. 1999; Vazquez and Pena de Ortiz
113 2004). In animal models lead induced activation of ERK1/2, and p38 (MAPK) signaling
114 (Cordova et al. 2004; Leal et al. 2006) in cerebellum of catfish and hippocampus of brain,
115 respectively. Neuronal differentiation and maturation genes, are targeted by low lead
116 exposure ([BPb] $< 10 \mu\text{g/dL}$) during maturation of retinal neurons (Chx10, Otx2, PKC-
117 alpha, VGlut1 and PMCA genes in rod photoreceptor and bipolar neurons) (Chaney et al.
118 2016) in GLE murine model and in adult brain neurons (Ngn1, Bdnf, Grin1, Grin2D, Grik5,

119 Gria4, and Grm6 genes in hippocampal neurons and embryonic stem cell derived
120 neurons) (Sanchez-Martin et al. 2013). Initial studies to determine if cell cycle progression
121 and differentiation of RPCs was altered in low lead exposure ([BPb] < 10 µg/dL) in retinas
122 showed increased incorporation of M-phase and S-phase markers, PH3 and BrdU in
123 RPCs and increased number of differentiated neurons (rod photoreceptors and bipolar
124 cells) from GLE mice (Giddabasappa et al. 2011). However, a quantitative measurement
125 of the individual phases of the cell cycle and cell fate specification, and the molecular
126 mechanism underlying this novel phenotype of increased RPC proliferation and neuronal
127 differentiation by GLE was not determined. Retina and brain are both parts of the central
128 nervous system. Thus, assessing the molecular mechanisms of how GLE effects RPC
129 proliferation and differentiation will be advantageous to gain generalized insights and
130 counter the detrimental effects of lead exposure on the developing central nervous
131 system of children.

132 In the developing mouse retina, six neuronal cell types and the Müller glial cell are
133 generated from a common pool of multipotent RPCs during a two-week period beginning
134 on embryonic day 11 (Cepko 2014). During retinal development, the RPCs proliferate and
135 differentiate into different cell types in a fixed histogenic order such that retinal ganglion
136 cells, cone photoreceptors, horizontal cells and most amacrine cells are born prior to birth
137 (early-born cells) and then rods, bipolar cells and the Müller glial cell are born postnatally
138 (late-born cells) (Cepko 2014; Marquardt and Gruss 2002; Young 1985b). Together, the
139 late-born rods (70%), bipolar cells (7-8%) and Müller glial cells (2-3%) comprise ~80% of
140 the total number of retinal cells (Jeon et al. 1998; Rapaport et al. 2004). During cell cycle
141 progression, the RPCs undergo interkinetic movement, during which S-phase RPC cell
142 bodies move from the inner retina or inner neuroblast layer (INBL) to the outer retina or
143 outer neuroblast layer (ONBL) for M-phase RPC cell bodies, while G1-phase and G2-
144 phase RPC cell bodies remain in between these layers (Baye and Link 2008). At the time
145 of mitosis, RPCs can divide in a symmetric or asymmetric manner and redistribute the
146 cell intrinsic factors, allowing for daughter RPCs to take similar or different developmental
147 paths of cell cycle reentry or cell cycle exit followed by differentiation (Baye and Link
148 2007). As most of the differentiated cells (neurons and glial cells) are gradually generated
149 postnatally from the proliferating RPCs whose cell cycle phase specific location is known,
150 postnatal RPCs are a great system to study the cellular dynamics and molecular
151 regulation of the competing processes of cell cycle progression, cell cycle exit and
152 neuronal-glial cell fate specification or differentiation of RPCs in a cell cycle phase specific
153 manner.

154 The essential molecular switch for cell cycle entry and progression is phosphorylation
155 of Retinoblastoma (RB), and other protein by a family of cyclin dependent kinases
156 (CDKs), CDK 2, 4, 6. Phosphorylated RB releases the transcription factor E2F from the
157 RB complex and is required for transcription of E2F cell cycle target genes. Regulation of
158 cell cycle is achieved by activation and inhibition of CDK kinase activity (Sherr and
159 Roberts 1999). These key regulators of CDKs are cyclins (Cyclins D, E, A and B) that
160 activate kinase activity of CDKs upon binding, while CDKs are inhibited by binding of
161 cyclin dependent kinase inhibitors (CKIs) of the Ink4 family (p15INK4b, p16INK4a,
162 p18INKc and p19INK4d) and the Kip family (p21CIP1, p27KIP1 and p57KIP2)
163 (Malumbres and Barbacid 2001). This dual CDK activity regulatory mechanism ensures
164 tight control of initiation, progression, termination and length of cell cycle, and its phases.

165 Increased activity of CDKs shortens cell cycle length and promotes cell cycle progression,
166 while decreased activity of CDKs lengthens cell cycle length and promotes cell cycle exit
167 (Alexiades and Cepko 1996; Livesey and Cepko 2001). During terminal cell cycle exit for
168 RPC differentiation, Kips family show additional specialization and 90% of the RPCs
169 utilize and upregulate p27KIP1 postnatally and p57KIP2 prenatally (Dyer and Cepko
170 2001).

171 Upon exit from cell cycle RPCs choose wither a neuronal or glial cell fate. This choice
172 is tightly regulated by neuronal basis-helix-loop-helix (bHLH) and homeodomain (HD)
173 transcription factors, NOTCH1-Hes1 pathway and JAK/STAT pathway, which results in
174 activation one cell fate choice and inhibition of other choices, to ensure correct ratio of
175 neuronal and glial cell in the retina. The combination of bHLH transcription factors ASCL1
176 (MASH1), NEUROD1 and OTX2 HD transcription factor, actively promote rod
177 photoreceptor and bipolar cell neuronal cell fate genes, while inhibiting Muller glial cell
178 fate in RPCs (Ahmad et al. 1998; Hatakeyama and Kageyama 2004). Proneuronal bHLH
179 transcription factors, such as MASH1 block glial cell fate of RPCs by inducing expression
180 of HES6, which forms inactive protein heterodimers with HES1 that blocks the ability of
181 HES1 to transcribe its target genes (Bae et al. 2000; Gratton et al. 2003). MASH1, NGN1
182 and other proneural transcriptional factors can directly inhibit the gliogenic JAK/STAT
183 pathway and by sequestering the SMADs, which are coactivators of STAT3 and thereby
184 block the ability of STAT3 to activate its target genes (Sun et al. 2001; Vetter and Moore
185 2001). On the other hand, the combination of NOTCH1-DELTA pathway induced bHLH
186 transcription factor HES1 (Furukawa et al. 2000; Tomita et al. 1996) and CNTF/LIF
187 mediated JAK pathway induced STAT3 phosphorylation (Goureau et al. 2004; Rhee and
188 Yang 2010), actively promotes expression of genes necessary for RPCs to take a Muller
189 glial cell fate, while inhibiting a neuronal cell fate. Proglial transcriptional factors, HES1
190 and STAT3 directly inhibit proneuronal factors by HES1 mediated sequestering of MASH1
191 and proneuronal bHLH factor coactivator E47 (Sasai et al. 1992) and STAT3 mediated
192 transcription of ID1/4 genes, which form inactive heterodimers MASH1 and other
193 proneuronal factors, thus preventing induction of neuronal cell fate gene expression
194 (Fukuda and Taga 2005). Thus, the balance of cell cycle Cyclins and Inks/CDKs dictate
195 the cell cycle state of RPCs, while the balance of proneuronal transcription factors (bHLH
196 and HD) and proglial transcription factors (HES1 and STAT3) dictate the cell fate
197 specification choice of RPCs.

198 The stages of cell cycle reentry, cell cycle progression, cell cycle exit and cell fate
199 specification are thought to be coupled processes such that when RPCs transition
200 through these stages, the preceding step is required to be stalled before the next step
201 ensues. At the molecular level this translates to the prerequisite that the molecular
202 regulators of the preceding step are completely extinguished before upregulation of the
203 regulators of the next step. This is supported by the observation that during postnatal
204 retinal development expression cyclins are progressively downregulated (Barton and
205 Levine 2008), while expression of proneuronal differentiation factors MASH1 (Nelson et
206 al. 2009) and OTX2 (Koike et al. 2007) are upregulated. However, this distinct coupling
207 of developmental stages does not appear to be rigid and ectopic expression of stage
208 specific molecular regulators of can occur during transition of RPCs, allowing for a unique
209 opportunity of cross-talk and coordination between these stages. For example, ASCL1, a
210 regulator of neuronal cell fate specification expected to be upregulated after cell cycle exit

211 of RPCs, can be expressed in RPCs still in cell cycle (Brzezinski et al. 2011), while the
212 cell cycle regulator, RB positively promotes rod photoreceptor differentiation in the
213 postnatal retina (Zhang et al. 2004). In another intriguing example, NOTCH1-HES1
214 pathway which promotes glial cell fate specification of RPCs, promotes maintenance of
215 stem cells and proliferation of RPCs, while continuing to inhibit neuronal cell fate
216 specification (Jadhav et al. 2006a; Jadhav et al. 2006b; James et al. 2004). A common
217 feature of the developmental process cell cycle (cyclins, INKS), cell cycle exit (p27 KIP,
218 p57CIP) and cell fate specification (ASCL1, NEUROD1, HES1, HES6, STAT3) regulators
219 is that their expression levels can undergo quick turnover by coordinated induction of their
220 mRNA expression, protein phosphorylation and protein degradation (Freire et al. 2012;
221 Shimojo et al. 2008). It may be hypothesized that when the kinetics of turnover of the
222 regulators is shorter than the length of the developmental process they are strictly
223 expressed in a stage specific manner in RPCs, while longer turnover kinetics than the
224 length of the developmental process causes leakiness or ectopic expression of these
225 regulators in preceding and following developmental stages of RPCs opening greater
226 opportunity for cross-talk between the stages. A comprehensive study of the spatio-
227 temporal expression (mRNA and protein) of these regulators and measurement of cell
228 cycle dynamics is missing in the field and is required to gain insight into the extend of
229 coordination of cell cycle, cell cycle exit and cell fate specification regulators during retinal
230 development. Another layer of regulation and coordinated regulation arises from
231 specialization of the different family members of cell cycle and cell fate specification
232 regulators. Loss of Cyclin D1 decreases RPC cell cycle reentry and induced expression
233 of Cyclin E, but not Cyclin D2 rescues the phenotype (Das et al. 2009), while another
234 study showed specialized cis-regulatory feedback loops between rod photoreceptor and
235 bipolar neuronal cell fate specification transcription factors fine tune neuronal subtype
236 choice (Wang et al. 2014). Similar experiments of multigene loss of function and rescue
237 studies or other methods of multigene expression perturbation is required to gain
238 functional insight into cross-talk and synergy relationships, between other molecular
239 regulators of RPC transition through cell cycle, cell cycle exit and cell fate specification.

240 In this study, we sought to identify the molecular regulators and changes in cell cycle
241 dynamics involved in the novel GLE phenotype of increased RPC proliferation and rod
242 photoreceptor, and bipolar cell differentiation, and gain a better understanding of retinal
243 development process. Affymetrix gene expression array identified the specific molecular
244 changes associated with GLE induced increased proliferation and rod photoreceptor, and
245 bipolar cell differentiation. Cell cycle analysis showed that relative to control retinas, GLE
246 shortened G1-phase of cell cycle and increased the output of cell cycle reentry, cell cycle
247 exit and neuronal cell fate specification of RPCs, without any change in glial cell fate. We
248 measured cell cycle dynamics and determined spatiotemporal expression pattern of this
249 comprehensive list of GLE dependent and developmentally important cell cycle (Cyclins,
250 p16 p19 INKs), cell cycle exit (p27 KIP), and neuronal glial cell fate specification factors
251 (ASCL1, OTX2, HES1) in control and GLE retinas. We found that cell cycle inhibitors
252 (p16, p19 INKs, p27KIP) and cell fate specification factors (ASCL1, OTX2, HES1) appear
253 together in the SVZ and disappear in the inner retina, while cell cycle activators and
254 regulators of cell cycle progression (Cyclins) shows the reverse pattern. This suggests
255 that cell cycle exit, HES1 mediated cell cycle reentry and cell fate specification show a
256 stronger spatial coupling, while cell cycle progression is specially uncoupled, which may

257 be necessary to ensure these processes can occur smoothly. Our results reveal an
258 intricate network of developmental regulators under GLE regulation and show the relative
259 coordinated spatiotemporal pattern of appearance and disappearance of developmental
260 regulators in relation to cell cycle dynamics. This study increases our understanding of
261 the molecular regulation of developing nervous system and GLE effects on it, and will
262 help meet the challenges of detrimental effects of GLE on the developing nervous system
263 and other developmental disorders.

264

265

266 **METHODS**

267

268 **Code availability.** This work does not contain any codes.

269

270 **Animal model.** All experimental and animal care procedures complied with the
271 National Institutes of Health (NIH) Public Health Service Policy on the Humane Care and
272 Use of Laboratory Animals (NIH 2002), and were approved by the Institutional Animal
273 Care and Use Committee of the University of Houston. All animals were treated humanely
274 and with regard for alleviation of pain and suffering. In rodents, brain and retinal
275 development during the first postnatal week is equivalent to the last trimester of human
276 gestation (Dobbing and Sands 1979; Martins and Pearson 2008). The murine GLE model
277 (Fig. 1a) used has been previously described (Chaney et al. 2016; Giddabasappa et al.
278 2011). Briefly, naive C57BL/6 wild-type female mice were given tap water for drinking
279 containing 0 ppm lead (Control group) or 55 ppm lead acetate (GLE group) for two weeks
280 prior to mating, during mating and pregnancy, and until PN10, after which both groups
281 were given lead free tap water. This treatment regimen ensured a steady lead level in
282 the GLE pups, with peak [BPb] on PN0 and/or PN10 of $22.11 \pm 1.05 \mu\text{g/dL}$, while control
283 group remained at [BPb] $0.75 \pm 0.06 \mu\text{g/dL}$ (Leasure et al. 2008). There were no statistical
284 differences between control and GLE groups on litter measure or body weight (Leasure
285 et al. 2008).

286

287 **Tissue processing, immunohistochemistry and confocal microscopy.** All tissue
288 processing, immunohistochemistry and confocal technique was performed essentially
289 as described (Chaney et al. 2016; Giddabasappa et al. 2011). Briefly, eyes were
290 removed and immersion fixed in 4% paraformaldehyde for 30min. Central retina 10um
291 sections were cut from cryoprotected frozen retinas at 200-400um distance from optic
292 nerve for confocal microscopy and slide mounted. Nuclear dyes and primary antibodies
293 against retinal development regulators were used at concentrations as listed in
294 Supplement Table 1. Mounted sections were post-fixed with 4% paraformaldehyde for
295 15min and treated with 1% sodium-borohydride to reduce auto-fluorescence. The
296 sections were washed in PBS, blocked with blocking buffer (10% normal goat serum
297 and 0.3% Triton-X100) and incubated for 48 hours at 4°C in primary antibody solution
298 prepared in blocking buffer. After PBS washes Alexa fluorescent, secondary antibodies
299 (1:400) made in blocking buffer were applied to the slides for 1 hour at room temperature
300 in the dark. The sections were washed, treated with DRAQ5 and coverslipped using
301 Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Confocal
302 images were taken using identical exposure and scanning parameters with a Leica TCS

303 SP2 LSCM (Leica Microsystems, Exton, PA). NIH ImageJ software was used to count
 304 colabeled cells at 63X magnification using the “analyze particles” protocol as previously
 305 described with a minimal particle size of 30 pixels. Mean \pm SEM values from these
 306 counts were used on the images and/or used on the graphs.

307
 308 **In vivo cell cycle dynamics measurements:** To assay cell cycle reentry and exit, cell
 309 fate specification, proliferation index and mitotic fraction we adapted for the retina
 310 previously described protocols of BrdU injection, immunohistochemistry and confocal
 311 microscopy (Chenn and Walsh 2002; Hodge et al. 2004; Young 1985a). Briefly, one
 312 dose of BrdU (100 μ g/gm body weight) was intraperitoneally (ip) administered into pups.
 313 In pups sacrificed 24 hours post-BrdU injection, fraction of MCM6+BrdU+ cells, MCM6-
 314 BrdU+ cells and OTX2+BrdU+ cells relative to total BrdU+ cells were computed using
 315 confocal microscopy to determine cell cycle reentry, cell cycle exit and cell fate
 316 specification indices, respectively. In pups sacrificed 45min post-BrdU injection, RPC
 317 proliferation index at PN1 was calculated as the fraction of MCM6+BrdU+ cells relative
 318 to total MCM6+ cells.

319 To assay cell cycle length, we essentially adapted for the retina previously
 320 published protocols (Hodge et al. 2004; Nowakowski et al. 1989). PN1 pups were
 321 injected with BrdU (100 μ g/gm body weight) intraperitoneally (ip), and sacrificed 2.5, 3.5
 322 and 4.5 hours following the injection. From confocal images the G2+M phase length
 323 was determined as the time required to colabel all PH3+ cells with BrdU+ such that the
 324 mean \pm SEM mitotic fraction equaled 1.0 ± 0.5 . To determine the total cell cycle length
 325 (TC) and total S phase length (TS) BrdU (100 μ g/gm body weight) was injected
 326 intraperitoneally (ip) into PN1 pups every 4 hours for 24 hours, and sacrificed 45
 327 minutes following the last injection. From confocal images a labeling index (LI) was
 328 calculated as the proportion of Draq5+ cells that were BrdU+ at each sacrifice time. The
 329 mean \pm SEM LI vs post-injection survival time was plotted for control and GLE retinas
 330 and the best-fit line for each was determined. The maximum LI value attained was the
 331 GF, while TC and TS were determined using the following equations: 1) y-intercept =
 332 GF X TS/TC, 2) time taken to reach maximum LI = TC - TS and 3) slope = 1/Tc. At the
 333 LI curve inflexion time and plateau, GF values were determined as the proportion of
 334 MCM6+, Draq5+ of total Draq5+ cells.

335
 336 **Affymetrix gene expression array and heat map.** Expression profiling experiments
 337 were performed at the Microarray Core Facility at Kellogg Eye Center, University of
 338 Michigan. Affymetrix gene expression array was performed following protocol provided
 339 by Affymetrix, Inc. (Santa Clara, CA) and as described before (Rueda et al. 2016; Zacks
 340 et al. 2006). Total RNA was isolated with Trizol and 2 to 5ug of total RNA was used as
 341 starting material for generation of biotinylated-cRNA (complementary RNA) with
 342 Affymetrix target labeling kit. The biotinylated-cRNA was hybridized to GeneChip Mouse
 343 Genome 430 2.0 array and scanned on Affymetrix Gene Chip Scanner 3000 7G. Robust
 344 Multichip Average (RMA) algorithm was used to obtain background corrected, quantile
 345 normalized, log₂ transformed expression values. Differentially expressed genes with a
 346 with mean expression values in log₂ scale ≥ 5 in one of the conditions and fold-change
 347 between GLE and control ≥ 2 , and p value was <0.05 were selected as GLE target
 348 genes. From the list of GLE targets, genes that classified into NIH-DAVID Gene

349 Ontology (GO) categories of “cell cycle” and “cell differentiation” were selected as
350 candidate genes for further analysis. Heat map visualization of these select candidate
351 GLE target genes was done using online tool <http://www.chibi.ubc.ca/matrix2png/>.
352

353 **RNA Isolation and qPCR.** Total RNA was extracted from retinas with Trizol reagent
354 (Invitrogen-Gibco) and cDNA was synthesized from 1ug of total RNA using oligo dT,
355 and random hexamers with iScript cDNA synthesis kit (Bio-Rad Cat. No. 170-8891).
356 Intron spanning primers designed with default settings on Roche Universal Probe
357 Library Design Center. List of primers are presented in Supplement Table 2. qPCR was
358 performed on the cDNA with SYBR green master mix buffer (Bio-rad) and 300 nM of
359 each forward, and reverse primers in a total volume of 25ul on Bio-Rad iCycler platform
360 (Bio-Rad Laboratories, Hercules, CA). After a denaturation step of 3 min
361 at 95°C, two-step amplification conditions were 40 cycles of 30 s at 95°C and 30 s at
362 60°C. Relative gene mRNA expression was calculated by using the comparative
363 threshold cycle ($\Delta\Delta C_t$) method with β -Actin as the internal normalization control. Gene
364 expression in GLE relative to control or fold change was computed using $2^{\Delta\Delta C_t}$.
365

366 **Western Blotting.** Whole cell protein was extracted from retinas with homogenization in
367 lysis buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM
368 Na_3VO_4 , 0.25% NP-40, 30 mM NaF, 60 mM β -glycerophosphate, 20 mM sodium
369 pyrophosphate, 1 protease inhibitor tablet, Roche Diagnostics, 10 mM Tris pH7.4).
370 Around 20-25 μg of total protein was loaded on 8% SDS-polyacrylamide gel and
371 transferred onto PVDF membrane. Blots were washed, blocked in blocking buffer (5%
372 milk in TBS-T) and probed with phospho and non-phospho antibodies prepared in
373 blocking buffer overnight at 4°C. Antibody concentrations are listed in Supplement Table
374 1. GAPDH was used as the loading control for all gels. Membranes were washed and
375 probed with HRP-conjugated secondary antibodies (1:10,000 dilution) prepared in
376 blocking buffer and imaged with chemiluminescence (ECL Plus: Amersham
377 Biosciences, Piscataway NJ). Non-saturated blots were scanned at high-resolution (600
378 dpi) for densitometry analysis using Adobe Photoshop CS.
379

380 **ChIP -qPCR.** Retinas were cross-linked with 1% formaldehyde for 15 min at room
381 temperature and cross-linking was quenched by adding 125mM glycine. Retinas were
382 homogenized in cold whole cell lysis buffer containing 0.5% NP-40 for 15 min at 4°C.
383 Cell debris was removed and nuclei collected by centrifugation at 13,000 rpm for 10
384 min. Nuclear pellet was resuspended in 1% SDS sonication buffer. Chromatin was
385 sonicated at 4°C using Fisher Scientific Sonic Dismembrator Model 500, for 10 sec x 4
386 times at 10% power. Sonicated samples were immunoprecipitation (IP) with 1ug
387 antibody conjugated Dynabeads Protein A (Invitrogen) overnight at 4°C. A list of
388 antibodies used is provided in Supplement Table 1. Immunoprecipitated DNA was
389 eluted from antibody-bead complex after washing. Eluted ChIP DNA and Input DNA
390 were reverse cross-linked overnight at 65°C with 192 mM NaCl and treated with
391 Protease K at room temperature for 1 hour . DNA was purified using phenol–
392 chloroform–isoamylalcohol extraction and was analyzed by qPCR (described above)
393 with primers spanning the promoter regions of CyclinD1 and Mash1 (Supplement Table
394 2). ΔC_t values were obtained from four replicates normalized against the C_t value of the

395 input DNA. The % Input for each ChIP fraction for the TFs were calculated as % Input =
 396 $2^{-\Delta\text{Ct}} [\text{normalized ChIP}] \times 100$.

397
 398 **Statistical Analysis.** For all confocal experiments four to five retinas were used, each
 399 from a different mouse litter at each age (PN 2, 4, 6 and 10) for control and GLE.
 400 Retinas from five to seven littermate pups were pooled per sample for each time point
 401 per treatment group for Affymetrix, qPCR, Western blot and ChIP-qPCR experiments.
 402 Data were analyzed by using ANOVA followed by post hoc multiple comparisons using
 403 Fisher's Least Significant Difference Test or the Student's T-test when only two means
 404 were compared. For all experiments, P-value lower than 0.05 was considered significant
 405 (*P<0.05). Significant differences between GLE and control are indicated with an *
 406 asterisk in figures. Graphs were generated with KaleidaGraph 4.0 (Synergy Software,
 407 Reading PA). Data are represented using mean \pm SEM.

408
 409

410 RESULTS

411 Time dependent and GLE dependent changes in number of RPCs reentering cell 412 cycle and exiting cell cycle with neuronal cell fate.

413 We wanted to determine the cell cycle and differentiation dynamics of RPCs
 414 during RPC development. Previously, we reported the GLE retinal phenotype of
 415 increased RPC proliferation, without any change in apoptosis, which resulted in an adult
 416 retina with more late-born rod and bipolar neuronal cells and no leftover RPCs
 417 (Giddabasappa et al. 2011). We hypothesized that GLE either simultaneously or
 418 sequentially increases both cell cycle reentry and neuronal cell fate specification during
 419 postnatal development. To test this hypothesis, we scored the number of RPCs
 420 reentering cell cycle (BrdU+MCM6+), exiting cell cycle (BrdU+MCM6-) and undergoing
 421 cell fate specification (BrdU+OTX2+), by staining sections 24 hours after a single BrdU
 422 pulse of BrdU (Fig. 1; Supp. Fig. 1). We found that At PN2 and PN4, $41.5 \pm 5.4\%$ and
 423 $65.3 \pm 15.7\%$ more RPCs reentered cell cycle (BrdU+MCM6+), respectively (Fig. 1b,d;
 424 Supp. Fig. 1a). RPCs exiting cell cycle (BrdU+MCM6-) from PN2 to PN6 increased by
 425 $38.0 \pm 3.5\%$ at PN2, $53.9 \pm 15.1\%$ at PN4 and $161.9 \pm 16.0\%$ at PN6 (Fig. 1b,e; Supp.
 426 Fig. 1a), an increase accompanied by a 40-50% increase in RPCs undergoing cell fate
 427 specification into rod-bipolar neurons (BrdU+OTX2+) (Fig. 1g,i; Supp. Fig. 1b). In both
 428 GLE and control retinas, the proportion of RPCs reentering cell cycle decreased with
 429 age (Fig. 1b,f; Supp. Fig. 1a), while the proportion of RPCs exiting cell cycle and
 430 undergoing cell fate specification increased with age (Fig. 1b,f,g,j; Supp. Fig. 1a,b).
 431 There was no difference in these ratios between GLE and controls.

432 The pattern of cell cycle reentry and cell cycle exit of RPCs in control is
 433 consistent with previous studies (Young 1985a, b), and additionally we show that the
 434 cell cycle exit pattern overlaps with rod photoreceptor and bipolar cell fate specification.
 435 In GLE we find simultaneous increases in number of RPCs undergoing cell cycle
 436 reentry, cell cycle exit and cell fate specification to rod photoreceptors, and bipolar
 437 neurons. However, GLE does not change the ratio of RPCs undergoing cell cycle, cell
 438 cycle exit and cell fate specification, which suggests that GLE does not effect the
 439 histogenic order of normal retinal development.

440

441 **GLE accelerates cell cycle progression of RPCs by shortening length of G1-phase**
 442 **of cell cycle.**

443 To determine the effect of GLE on cell cycle kinetics further, we measured total
 444 cell cycle length and length of cell cycle phases. For a quick assessment of cell cycle
 445 length change, we labeled PN1 RPCs with a single 45min pulse of BrdU and found GLE
 446 increases proliferation index or proportion of MCM6+BrdU+ (Supp. Fig. 1c, control $29 \pm$
 447 7% , GLE $48 \pm 9\%$). This shows that GLE shortens cell cycle, so we specifically
 448 measured the length of different phases of cell cycle to determine the exact phase or
 449 phases that were shortened by GLE. PN1 control and GLE retinas were injected with a
 450 single pulse of BrdU and sacrificed at different times, and BrdU colabeling with PH3+
 451 mitotic marker was scored. The results showed that the combined length of the G2+M
 452 phases of cell cycle was invariant at T_{G2+M} 4.5 hours in both GLE and control retinas
 453 (Supp. Fig. 1d). To determine the total cell cycle length and length of other phases of
 454 cell cycle we utilized cumulative BrdU-labeling. Plots of the LI vs survival time for both
 455 control and GLE illustrated that labeling index (LI) increases linearly to a maximum
 456 value and then levels off (Fig. 1k). The relationship between LI and survival time was
 457 used to calculate the cell cycle length parameters. In GLE relative to control, the
 458 calculated value of T_c was shorter by 7.6 hours or 24% (31.9 ± 3.7 hours in control,
 459 24.3 ± 0.9 hours in GLE), but T_s (11.4 ± 3.6 hours in control, 11.6 ± 2.5 hours in GLE)
 460 and GF (0.57 ± 0.02 in control, 0.57 ± 0.03 in GLE) were not different (Fig. 1k). The
 461 composite results from the cell cycle length experiments and G2+M phase analysis
 462 enable the determination of T_{G1} [$G1 = T_c - T_s - (G2+M)$]. In GLE retinas relative to
 463 control, the T_{G1} was 7.8 hours shorter or almost 50% less accounting for the overall
 464 decrease in cell cycle length (16.0 ± 1.9 hours in control, 8.2 ± 0.4 hours in GLE).

465 The control retina cell phase lengths are consistent with previously published
 466 results, T_c 31.9 ± 3.7 hours, T_{G1} 16.0 ± 1.9 hours, T_s 11.4 ± 3.6 hours and T_{G2+M} 4.5
 467 hours (Young 1985a). Only, T_{G1} was shorter in GLE by 7.8 hours. Taken together the
 468 analysis of cell cycle dynamics in GLE shows that GLE accelerated cell cycle and
 469 increase the number of RPCs reentering cell cycle, and exiting cell cycle with neuronal
 470 cell fate specification.

471
 472 **GLE regulates cell cycle and neuronal differentiation gene network.**

473 This GLE effect contrasts with normal development, during which shortening of
 474 cell cycle length increases number of RPCs reentering cell cycle at the expense of
 475 RPCs exiting cell cycle and differentiating (Julian et al. 2016; Miles and Tropepe 2016).
 476 Therefore, we endeavored to understand the molecular mechanism of this unique GLE
 477 phenotype whereby GLE shortened cell cycle, increased cell cycle reentry and yet
 478 increased cell cycle exit and differentiation. Therefore, we performed Affymetrix gene
 479 expression array in GLE and control retinas during postnatal development. We selected
 480 the postnatal development period because GLE accelerated cell cycle and increased
 481 rod-bipolar cell fate specification postnatally. A total of 822 genes were differentially
 482 expressed between GLE and control retinas (Fig. 2a). To begin to understand the
 483 biological function of these genes we performed NIH DAVID Gene Ontology (GO)
 484 analysis, which revealed “sensory perception of light stimulus”, “nervous system
 485 development”, “cell cycle” and “neuronal differentiation” as major GOs (Fig. 2a). As GLE
 486 increased cell cycle and neuronal cell fate specification for further validation and

487 analysis we focused on the genes in the “cell cycle” (includes cell cycle exit gene p27
488 Cdkn1b) and “neuronal differentiation” GOs (Fig. 2b). The differential expression of
489 these genes in GLE supported their functional and physiological relevance. We
490 predicted that this comprehensive study of the spatiotemporal expression of these
491 candidate regulators of cell cycle progression, cell cycle exit and cell fate specification,
492 and the cell cycle dynamics measurements made in this study will reveal new insights
493 into the coupling and uncoupling of these processes in RPCs during normal
494 development.

495 We used qPCR to examine the expression of these GLE target candidate genes,
496 cell cycle/cell cycle exit genes (*CyclinD1*, *CyclinE2*, *CyclinA2*, *Mcm6*, *Cdk2*, *Cdk4*, *Rb*,
497 *p107*, *E2f1*, *Notch1*, *Hes1*, *p27 Cdkn1b*, *p16 Cdkn2a* and *p19 Cdkn2d*) and neuronal
498 differentiation genes (*Hes6*, *Mash1*, *Otx2* and *Neurod1*). In the cell cycle group, the
499 temporal pattern of gene expression of cyclins (*CyclinD1*, *CyclinE2* and *CyclinA2*), cell
500 proliferation marker (*Mcm6*), cell cycle kinases (*Cdk2*, *Cdk4*) and Notch pathway
501 transcription factors (*Hes1*, *Hes6*) decreased with age consistent with completion of
502 developmental cell cycle and were significantly upregulated in GLE relative to control by
503 39% or more (Fig. 2c,d,e,f,g,h,i,j). Cell cycle inhibitors (p16 Cdkn2a, p19 Cdkn2d)
504 showed a temporal expression pattern similar to cyclins, but were significantly
505 downregulated in GLE by 24% (Fig. 2k,l). In the cell cycle exit and differentiation gene
506 group, expression of cell cycle exit factor *p27 Cdkn1b*, cell cycle inhibitor and rod
507 differentiation factor *Rb1*, rod photoreceptor and bipolar neuronal differentiation factors
508 (*Otx2*, *Mash1* and *Neurod1*) in control and GLE retinas, peaked mid-way of postnatal
509 development at PN6. Moreover, in GLE *Otx2*, *Mash1*, *Rb1* and *p27 Cdkn1b* were
510 significantly upregulated relative to control by 30% or more (Fig. 2m,n,o,p,q).

511 Molecular coupling of cell cycle exit and cell fate specification, and molecular
512 uncoupling of these processes with cell cycle reentry in RPCs during development is
513 suggested by the expression pattern of cell cycle regulators (cyclins, Inks, Cdks,
514 *Mcm6*), which closely overlay with RPC cell cycle reentry temporal pattern (Fig. 1d), and
515 expression pattern of regulators of cell cycle exit and differentiation overlap with RPC
516 cell cycle exit and cell fate specification temporal pattern (Fig. 1e,i). For GLE these
517 results suggest, GLE mediated upregulation of cell cycle activators (cyclins, Cdks,
518 *Mcm6*, *Hes1*), downregulation of cell cycle inhibitors (Inks), cell cycle exit (p27) and
519 neuronal cell fate specification factors (*Ascl1*, *Otx2*, *Hes6*), underlies and is consistent
520 with the increased cell cycle reentry, cell cycle exit and neuronal cell fate specification of
521 RPCs in GLE.

522 To gain greater developmental insight into spatiotemporal coupling and
523 uncoupling or separation of regulators of cell cycle reentry, cell cycle exit and cell fate
524 specification we performed immunohistochemistry. Moreover, we performed western
525 blot analysis to quantify the protein expression levels and phosphorylation activation
526 status of these molecular regulators. These results in control retina was compared with
527 GLE to gain insights into the spatiotemporal and protein level GLE mediated changes to
528 molecular regulators of cell cycle reentry, cell cycle exit and cell fate specification. In
529 immunohistochemistry, RPCs were labeled with proliferation markers MCM6 or Ki67.
530 These results and their conclusions are described below.

531

532 **Spatiotemporal expression of CYCLINs during development is restricted to INBL**
 533 **during development and GLE upregulates expression of CYCLINs in RPCs.**

534 Cell cycle progression requires sequential expression of CYCLINs that
 535 sequentially activate CDK phosphorylation activity (Levine and Green 2004). We found
 536 that CYCLIN D1 and CYCLIN A2 were localized in RPCs from PN2 to PN8. Their
 537 highest expression was at PN2 and labeled cells were present throughout the ONBL,
 538 but by PN6 with increasing age labeled cells were more pronounced in the proximal
 539 ONBL and INBL, the site of S phase, and gradually decreased until the SVZ, the site of
 540 M phase (Fig. 3a,b; Supp Fig. 2a,b). From PN2 to PN6 50-92% and 40-67% of the
 541 MCM6+RPCs colabeled with CYCLIN D1⁺ and CYCLIN A2⁺, respectively (Fig. 3a, b;
 542 Supp Fig. 2a,b). The difference between the GLE retinas relative to controls, was that
 543 the number of MCM6 and CYCLIN D1 or CYCLIN A2 colabeled cells increased by 27%
 544 to 68% (Fig. 3a,b; Supp Fig. 2a,b). Western blots and densitometry from PN2 to PN10
 545 also showed an age dependent decrease in expression of CYCLIN D1, CYCLIN E2 and
 546 CYCLIN A2, and these cyclins were upregulated in GLE by 28-39% (Fig. 3c,d,e,f).

547 These result show that the expression of CYCLINs is predominantly in the INBL,
 548 the site of S-phase and the spatiotemporal expression pattern is similar to the laminar
 549 distribution of RPCs (MCM6+ cells), consistent with previous studies(Barton and Levine
 550 2008). Both mRNA and protein expression of Cyclins is the same as the pattern of RPC
 551 cell cycle reentry (Fig. 1d). In GLE relative to control, the spatiotemporal expression
 552 pattern of CYCLINs is not altered, but an increased number RPCs express the
 553 CYCLINs, and the mRNA and protein expression of CYCLINs is higher in GLE, and is a
 554 molecular mechanism consistent with the GLE mediated accelerated cell cycle,
 555 increased cell cycle reentry, and cell cycle progression of RPCs.

556
 557 **CDK activity and RB1 hyperphosphorylation closely follows CYCLIN expression**
 558 **and RPC cell cycle entry pattern, and are upregulated in GLE.**

559 Upregulation of cyclins are directly linked with increased activation of CDKs and
 560 subsequent hyperphosphorylation of RB which induces cell cycle reentry and
 561 progression by relieving E2F1 repression by RB1 (Bilitou and Ohnuma 2010). We found
 562 an age dependent decrease in expression of total CDKs (CDK4 and CDK2) and
 563 activated CDKs (pCDK2), proliferation marker (MCM6), RB and hyperphosphorylated
 564 RB1, p107 (RB family member) and E2F1 (Fig. 3c,g,h,i,j,k,l; Supp. Fig. 2f,g). These
 565 result show that both mRNA and protein expression of these cell cycle regulators,
 566 activity of CDKs and hyperphosphorylation is the same as the pattern of RPC cell cycle
 567 reentry (Fig. 1d). In GLE relative to control, we found an upregulation of expression
 568 (indicated in parentheses) in GLE of MCM6 (28-34%), CDK4 (34-41%), CDK2 (23-
 569 28%), RB1 (20-38%), activated CDK2 (pCDK2) (20-36%) and hyperphosphorylated
 570 RB1 (36-52%) (Fig. 3c,g,h,i,j,k,l; Supp. Fig. 2f,g). Thus, consistent with GLE induced
 571 increased expression of CYCLINs, there is increases activity of the cyclin dependent
 572 effector kinases, CDKs that in turn hyperphosphorylates RB1, an essential step for cell
 573 cycle progression, and is a molecular mechanism consistent with the GLE mediated
 574 accelerated cell cycle, increased cell cycle reentry, and cell cycle progression of RPCs.

575
 576

577 **Spatiotemporal expression of INKs during development is restricted to SVZ during**
 578 **development and GLE downregulates expression of INKs in RPCs.**

579 p19 INK4d inhibit the activity of CDKs and are integral to cell cycle progression
 580 (Levine and Green 2004). We found that p16 INK4a+ (CDKN2A) and p19 INK4d+
 581 (CDKN2D) spatiotemporal expression pattern was highest in the SVZ, where cells
 582 undergo mitosis, and the distal ONBL (Fig. 4a,b; Supp. Fig.2c,d). From PN2 to PN6 only
 583 14% of the MCM6+ cells colabeled with p16 INK4a (Fig. 4a; Supp. Fig.2c). The age-
 584 dependent decrease in p16 INK4a and p19 INK4d, and their expression in RPCs is
 585 consistent with the age-dependent decrease in cell cycle. In GLE retinas the number of
 586 MCM6+RPCs colabeled with p16 INK4a decreased by 30%-52% relative to control (Fig.
 587 4a; Supp. Fig.2c). The spatiotemporal expression of p19 INK4d appeared lower in GLE
 588 retinas compared to controls (Fig. 4b; Supp. Fig.2d). Western blot and densitometry
 589 from PN2 to PN10, confirmed the decreased expression of p16 INK4a and p19 INK4d
 590 with age in both GLE and control, and the downregulation of these genes in GLE by 20-
 591 27% relative to control (Fig. 4d,e,f). This shows that decreased p16 INK4a and p19
 592 INK4d expression in RPCs by GLE underlies the molecular mechanism of increased cell
 593 cycle reentry and cell cycle progression of RPCs.

594 These result show that the expression of INKs is predominantly in the SVZ, the
 595 site of M-phase and the spatiotemporal expression pattern is opposite to the laminar
 596 distribution of CYCLIN+ RPCs in S-phase at the INBL (Fig. 3a,b). The spatiotemporal
 597 expression pattern of p19 is consistent to previous studies (Cunningham et al. 2002),
 598 while p16 spatiotemporal expression consistent had not been studies before. Both
 599 mRNA and protein expression of INKs is the same as the pattern of RPC cell cycle
 600 reentry (Fig. 1d), than cell cycle exit (Fig. 1e) and cell fate specification (Fig. 1i), which
 601 suggests that INKs may contribute more to cell cycle reentry. In GLE relative to control,
 602 the spatiotemporal expression pattern of INKs is not altered, but a decreased number
 603 RPCs express the INKs, and the mRNA and protein expression of INKs is lower in GLE,
 604 and is a molecular mechanism consistent with the GLE mediated accelerated cell cycle,
 605 increased cell cycle reentry, and cell cycle progression of RPCs.

606
 607 **p27 KIP1 is uniformly expressed spatiotemporal in SVZ and INBL during early**
 608 **development, and GLE upregulates expression of p27 KIP1 in RPCs.**

609 p27 Kip1, inhibits cell cycle of RPCs to promote cell cycle exit in over 90% of
 610 RPCs exiting cell cycle in postnatal retina (Levine et al. 2000). Thus, differential
 611 regulation of p27 Kip1 is an indicator and regulator of RPC cell cycle exit. At PN2, p27
 612 KIP1+ (CDKN1B+) cells were dispersed throughout the SVZ and ONBL, at PN4 they
 613 were highly expressed in the SVZ and distal ONBL, at PN6 they were in the SVZ, ONBL
 614 and INBL, and by PN8 they were exclusively located in Müller glial cells (Fig. 4c; Supp.
 615 Fig. 2e). At PN2 to PN6 only 28% of the MCM6+ cells colabeled with p27 KIP1 (Fig. 4c).
 616 Between GLE and control retinas the number of MCM6 and p27 KIP1 colabeled cells in
 617 GLE retinas, significantly increased at PN2 to PN6 by 126-87% (Fig. 4c; Supp. Fig. 2e).
 618 Western blot and densitometry from PN2 to PN10, showed that both control and GLE
 619 retinas had the same pattern of p27 KIP1 expression, but in GLE retinas p27 KIP1
 620 expression significantly increased by 37% relative to control (Fig. 4d,g).

621 The pattern of p27 KIP1 expression (peak at PN4) and its colabeling with MCM6
 622 is similar to the pattern of cell cycle exit, consistent with the role in promoting cell cycle

623 exit (Fig. 1e). At PN6 and PN8, the expression of p27 KIP1 intensifying in the INBL at
 624 the site of Müller glial cells location, consistent with previous results (Levine et al. 2000).
 625 The increased expression of p27 KIP1 and increased colabeling of p27 KIP1 with
 626 MCM6 is consistent with increased cell cycle exit in GLE. Thus, increase of p27 KIP1
 627 expression is the molecular mechanism by which GLE increases cell cycle exit of RPCs.
 628

629 **Spatiotemporal expression of neuronal cell fate specification factors (OTX2,**
 630 **MASH1 and HES6) during development is restricted to SVZ and GLE specifically**
 631 **upregulates their expression without altering glial cell fate specification factors.**

632 OTX2 and MASH1 are proneural factors that determine rod and bipolar cell fate
 633 (Nelson et al. 2009; Wang et al. 2014). From PN2 to PN4, the spatiotemporal
 634 expression of OTX2+ or MASH1+ and MCM6+RPCs colabeled cells were located in the
 635 outer retina in the SVZ and ONBL, and OTX2 appeared also in the INBL at PN6 (Fig.
 636 5a,b; Supp. Fig.3a,b). Western blot and densitometry, corroborated these results and
 637 showed, expression peak of OTX2, MASH1, HES6 and NEUROD1 at PN4 during retinal
 638 development (Fig. 5c,d,e,f,g). In GLE retinas from PN2 to PN6 relative to control, there
 639 was 36% and 40% increase in OTX2 and MASH1 colabeled MCM6+RPCs, respectively
 640 without any change in spatiotemporal pattern of expression (Fig. 5a,b; Supp. Fig.3a,b).
 641 In GLE retinas relative to control, protein expression increased for OTX2 (28-44%),
 642 HES6 (31-36%) and MASH1 (14-52%) from PN2 to PN10 (Fig. 5c,d,f,g).

643 The pattern of OTX2, MASH1, NEUROD1 and HES6 expression (peak at PN4)
 644 and its colabeling with MCM6 is similar to the pattern of neuronal cell fate specification
 645 (Fig. 1i), consistent with their role in promoting neuronal cell fate specification. At PN6
 646 and PN8, the expression of OTX2 intensifying in the INBL at the location of Muller glial
 647 cells has not been reported before and suggests potential non-neuronal functions of
 648 OTX2. Interestingly, the pattern of expression of cell cycle exit (Fig. 1e) and its regulator
 649 p27 KIP1 (Fig. 2e), and neuronal cell fate specification (Fig. 1i) and these neuronal cell
 650 fate regulators (OTX2 and MASH1) are spatiotemporally coupled, suggesting potential
 651 cooperation between these processes. In GLE relative to control, the increased
 652 expression of OTX2, MASH1 and HES6 and increased colabeling of OTX2 and MASH1
 653 with MCM6 is consistent with increased neuronal cell fate specification in GLE. Thus,
 654 increase of OTX2, MASH1 and HES6 expression is the molecular mechanism by which
 655 GLE increases rod-bipolar neuronal cell fate specification and differentiation of RPCs.
 656

657 **Developmental age dependent switch in expression of NOTCH1 and HES1 from**
 658 **SVZ to INBL, and GLE differentially modulates NOTCH1 and HES1 activity.**

659 The NOTCH1-HES1 pathway oscillatory expression promotes the proliferative
 660 expansion of the RPC pool and inhibits neuronal differentiation, whereas sustained
 661 expression of NOTCH1-HES1 pathway promotes glial cell fate (Balenci and van der Kooy
 662 2014; Mizeracka et al. 2013; Nelson et al. 2009). At PN2 and PN4, NOTCH1 and HES1
 663 expression was high in the SVZ and ONBL (Fig. 6a; Supp. Fig. 3c). From PN6 onwards,
 664 NOTCH1 and HES1 expression switched to high levels in INBL (Fig. 6a; Supp. Fig. 3c).
 665 The number of HES1 cells colabeled with RPCs significantly decreased from PN2 to PN8.
 666 In PN2 and PN4 almost all HES1+ cells colabeled with the Ki67+RPCs, 30% and 45% in
 667 control and GLE, respectively (Fig. 6a; Supp. Fig. 3c). By PN6, HES1+ and Ki67+
 668 colabeling dramatically decreased by 15% in GLE relative to control (Fig. 6a; Supp. Fig.

669 3c). Western blot and densitometry, also showed an age dependent decrease in
 670 NOTCH1, NOTCH1-ICD (activated Notch1) and HES1 (Fig. 6b,c,d,e). In GLE retinas
 671 relative to controls from PN2 to PN4 there was increase in NOTCH1-ECD (25%),
 672 NOTCH1-ICD (30%) and HES1 (30%) consistent with increased proliferation, and all
 673 three proteins expression decreased by ~20% at PN6 in GLE consistent with completion
 674 of proliferation and increased neuronal cell fate specification (Fig. 6b,c,d,e). NOTCH1-
 675 HES1 also potentiates Muller glial cell fate specification through STAT3 activation, so we
 676 performed Western and densitometry in control and GLE retinas for STAT3 and p-STAT3
 677 to determine if the activation of Notch1-Hes1 activated the glial pathway in GLE
 678 (Mizeracka et al. 2013). Both STAT3 and p-STAT3 (activated STAT3) expression
 679 increased from PN2 to PN4, and stayed high during the rest of the development. Between
 680 GLE and control there was no difference in STAT3 and p-STAT3 consistent with the fact
 681 that GLE does not change Muller glial cell fate (Fig. 6b,f,g).

682 These result show that the expression of NOTCH1-HES1 pathway is
 683 predominantly active in the SVZ, the site of M-phase. Both mRNA and protein
 684 expression of NOTCH1-HES1, and activated NOTCH1 (NOTCH1-ECD) is the same as
 685 the pattern of RPC cell cycle reentry (Fig. 1d), than cell cycle exit (Fig. 1e) and cell fate
 686 specification (Fig. 1i), which suggests that NOTCH1-HES1 pathway may contribute
 687 more to cell cycle reentry. Interestingly, there is no correlation in the pattern of
 688 NOTCH1-HES1 pathway and STAT3-pSTAT3 glial cell fate specification pathway,
 689 which suggests these pathways may have independent modes of glial cell fate
 690 specification in the postnatal retina. In GLE relative to control, the spatiotemporal
 691 expression pattern of NOTCH1-HES1 is not altered, but an increased number RPCs
 692 express the HES1, and the mRNA and protein expression of NOTCH1-HES1 is higher
 693 in GLE early on consistent with more cell cycle reentry, and lower in GLE later
 694 consistent with less cell cycle reentry and more neuronal cell fate specification, without
 695 change in Müller glial cell fate specification. Thus, NOTCH1-HES1 pathway modulation
 696 is a molecular mechanism consistent with the GLE mediated increased cell cycle
 697 reentry, and cell cycle progression of RPCs early on, and later with increased cell cycle
 698 exit and cell fate specification of RPCs. This shows that GLE mediated alteration in
 699 NOTCH1-HES1 pathway activity is a network hub of cross-talk between cell cycle and
 700 cell fate specification of RPCs.

701

702 **GLE increases activity of the Cyclin D1 and Mash1 gene promoters.**

703 We hypothesized that GLE increases recruitment of transcription factors at
 704 promoters of *Cyclin D1* and *Mash1* to upregulate their expression relative to control. To
 705 determine the transcriptional regulatory mechanisms underlying the GLE phenotype of
 706 simultaneously increased proliferation and rod photoreceptor, and neuronal cell fate
 707 specification, we investigated the recruitment of transcription factors by ChIP-qPCR on
 708 the *Cyclin D1* and *Mash1* promoter.

709 In both control and GLE retinas ChIP-qPCR showed that RNA POL2, E2F1 and
 710 C-JUN association at *CyclinD1* promoter was highest at PN2 and PN4, and decreased
 711 at PN6, (Fig. 7a,b,c). In GLE retinas there was increased recruitment of RNA POL2,
 712 E2F1, C-JUN and N-MYC relative to control (Fig. 7a,b,c). In control and GLE retinas,
 713 the relative binding of RNA POL2 and C-JUN to the *Mash1* promoter was highest at
 714 PN4, while that of E2F1 was highest at PN2 and PN4 (Fig. 7d,e,f). In GLE retinas

715 compared to controls, the relative binding activity of RNA-POL and C-JUN to the *Mash1*
 716 promoter increased from PN2-PN6, and E2F1 binding increased from PN2 to PN4 (Fig.
 717 7d,e,f).

718 These results show that during development *Cyclin D1* and *Mash1* share
 719 transcription factors (RNA POL2, E2F1, C-JUN, N-MYC), which provides a point of
 720 coordination between cell cycle and neuronal cell fate specification, and the occupancy
 721 of their promoters decreases at PN6 with exhaustion of RPCs and completion of cell
 722 cycle and cell fate specification. In GLE the increased binding of key transcription
 723 factors to the *CyclinD1* and *Mash1* promoters is consistent with increased proliferation
 724 and rod-bipolar differentiation .

725

726 DISCUSSION

727 This study has revealed more of the complexity of coordination of cell cycle, cell
 728 cycle exit and cell fate specification processes and their molecular regulators in RPCs
 729 during retinal development (Fig. 7g,h). We show several lines of evidence to show
 730 strong coupling between temporal pattern of number of RPC undergoing cell cycle exit
 731 and neuronal cell fate specification, and temporal expression pattern of molecular
 732 regulators of cell cycle exit (p27 KIP1) and neuronal cell fate specification (ASCL1,
 733 OTX2, NEUROD1, HES6). Temporal expression pattern of molecular regulators of cell
 734 cycle, the inhibitors (p16 INK4a, p16 INK4d), CDK activity (pRB, pCDK4), NOTCH1-
 735 HES1 pathway proteins and cyclins (CYCLIN D1, A2, E1) showed strong coupling with
 736 temporal pattern of number of RPCs undergoing cell cycle reentry. These results show
 737 that expression pattern is a good indicator of molecular function and RPC phenotypic
 738 outcome.

739 Timing of cell fate specification in RPCs by neuronal cell fate specification factors
 740 and the role of cell cycle inhibitors in cell fate specification in RPCs has been a long-
 741 standing question. The cell cycle inhibitors, p57KIP2 and p27KIP1 expression in RPCs
 742 induce cell cycle exit in embryonic and postnatal RPCs, respectively (Dyer and Cepko
 743 2001), while among INKs, p19INK4d expresses with p27KIP1 in RPCs to potentiate cell
 744 cycle exit (Cunningham et al. 2002). Additionally, p27KIP1 also promotes Müller glial
 745 cell fate of RPCs and p27KIP1 levels are maintained high in Müller glial cells (Levine et
 746 al. 2000), but if the INKs play any role in cell fate specification is unknown. Our results,
 747 show that p16INK4a and p19INK4d expression pattern is spatially localized with
 748 p27KIP1 and neuronal factors (OTX2, MASH1) in SVZ RPCs and postmitotic cells,
 749 which supports the possible role of INKs in cell cycle exit and cell fate specification in
 750 RPCs (Fig. 7g,h). The known mechanism of action of cell cycle inhibitors is by blocking
 751 CYCLIN-CDK complex kinase activity, but how p27KIP1 and potentially INKs regulate
 752 cell fate specification requires further study. Expression of neuronal factor NGN2 in
 753 subset of embryonic RPCs primes them for ganglion neuron cell fate, while MASH1
 754 expression in subset of embryonic RPCs primes them to other neuronal cell fates
 755 (Brzezinski et al. 2011). The potential mechanism of action of neuronal cell fate
 756 specification factors in RPCs is through transcriptional regulation, and it has been
 757 shown that MASH1 directly regulate cell cycle genes in addition to its canonical
 758 neuronal target genes and in embryonic brain and neural stem cell culture (Castro et al.
 759 2011). The RPC priming by neuronal cell fate transcription factors, such as MASH1 is
 760 different from classical cell fate specification that occurs in RPCs by expression of the

761 neuronal cell fate transcription factors. A primed RPC should in principle undergo one or
762 two rounds of cell cycle before cell fate specification terminal differentiation. Our results,
763 show that neuronal factors, MASH1 and OTX2 expression pattern is spatially localized
764 with p27KIP1 and INKs (p16 INK4a, p19INK4d) in SVZ RPCs and postmitotic cells (Fig.
765 7g,h). Importantly, we did not observe MASH1+ RPCs or OTX2+ RPCs in INBL, which
766 would have appeared had RPCs been primed and continued to cycle at least once
767 before cell fate specification. These results support the idea that MASH1 and OTX2
768 predominantly play a role in cell fate specification at the end of terminal differentiation in
769 postnatal RPCs and not in RPC neuronal cell fate priming. Another, possible
770 explanation is that in our studies which were in postnatal retina, MASH1 mediated RPC
771 priming does not occur, while priming of RPCs by MASH1 is a process that occurs in
772 embryonic retina the timepoint for the previous study that showed MASH1 mediated
773 RPC priming (Brzezinski et al. 2011). More research is required to resolve the context of
774 MASH1, OTX2 and other neuronal cell fate specification factors in RPCs.

775 The NOTCH1-HES1 pathway is the master positive regulator of proliferation and
776 glial cell fate, and actively inhibits neuronal cell fate (Zhou et al. 2010). In the present
777 study, we show that NOTCH1 and HES1 are spatially localized to the SVZ and ONBL in
778 RPC and postmitotic cells, similar to cell cycle inhibitors (p27KIP1, p16 INK4a, p19
779 INK4d) and neuronal cell fate specification factors (OTX2, MASH1), while cell cycle
780 activator cyclin expression was spatially separated localized to the INBL in RPC and
781 few postmitotic cells (Fig. 7g,h). During cell cycle the RPC cell bodies move between
782 INBL, site of S phase to SVZ, site of M phase, while G1 and G2 phases occur in
783 between SVZ and INBL (Baye and Link 2007). Taken together these results suggest
784 that the decisions for cell cycle reentry, cell cycle exit and cell fate specification are
785 being taken in the RPCs in the SVZ, and the molecular cascade regulating these
786 processes (p27 KIP, INKs, OTX2, MASH1) are potentially being inhibited by NOTCH1-
787 HES1 in RPCs to promote cell cycle reentry in RPCs. NOTCH1-HES1 pathway can
788 inhibit p27 KIP1 in different developmental tissues (Murata et al. 2005), MASH1 in
789 central nervous system (Kageyama and Ohtsuka 1999) and activate CYCLIN D1 in lens
790 (Rowan et al. 2008). Interestingly, we did not find a major NOTCH1-HES1 expression in
791 RPCs in INBL where CYCLINs are highly expressed in RPCs, which suggested that
792 once the RPC decide to reenter cell cycle under NOTCH1-HES1 regulation in SVZ and
793 ONBL, the regulation of cell cycle progression is regulated by CYCLINs and does not
794 require sustained expression of NOTCH1-HES1 in INBL. In future studies, it remains to
795 be seen if in the retina NOTCH1-HES1 pathway regulates INKs, OTX2 and other
796 neuronal specification factors, and CYCLINs. It is known that RPCs mainly divide
797 asymmetrically in postnatal retina (Kechad et al. 2012) and asymmetrically dividing
798 progenitor cells are known to asymmetrically distribute molecular regulators (Roegiers
799 and Jan 2004). At PN4 we observe almost equal number of RPCs reentering cell cycle,
800 and exiting cell cycle and undergoing cell fate specification. These results further
801 suggest that RPCs divide asymmetrically to generate two daughter cells, one rich in
802 NOTCH1-HES1, and another rich in cell cycle inhibitors (p27 KIP1, INKs) and neuronal
803 cell fate specification factors (MASH1, OTX2), resulting in a peak of both proliferating
804 RPCs and differentiating RPCs at PN4. As finding primary antibodies from different
805 species is challenging to do colabeling studies of all these regulators, it is hoped that in
806 future single cell RNA-seq of RPCs from PN4 retinas, to understand the frequency of

807 combination of these and other molecular regulators that RPCs utilize to undergo cell
808 cycle reentry, cell cycle exit and cell fate specification.

809 In this study, we have found a novel molecular mechanism of cell cycle, cell cycle
810 exit and cell fate specification factors (Fig. 7g,h) underlying the previously reported
811 novel GLE phenotype of increased proliferation and neuronal cell fate specification in
812 the retina, without change in apoptosis (Giddabasappa et al. 2011). The cellular
813 mechanism to cause this novel GLE phenotype could be sequential or concurrent
814 increase in RPC proliferation and RPC differentiation into rod photoreceptor and bipolar
815 neurons. Analysis of cell cycle dynamics and differentiation dynamics of RPCs using
816 BrdU and other cellular markers, we found that GLE induced a concurrent increase in
817 RPCs reentering cell cycle, and undergoing cell cycle exit and cell fate specification or
818 differentiation. It is known that during normal development, shortening of cell cycle
819 length causes increase in cell cycle reentry of RPCs at the expense of cell fate
820 specification (Alexiades and Cepko 1996; Takahashi et al. 1993). Surprisingly, we found
821 that GLE accelerated cell cycle by shortening the length of G1 phase of cell cycle
822 without changing the ratio of RPCs undergoing cell cycle reentry and cell cycle exit, and
823 cell fate specification. This raised the question, how does GLE balance the normal
824 developmental force of decreased cell fate specification of RPCs with an increase in cell
825 cycle reentry of RPCs when shortening of cell cycle length occurs. The G1 phase
826 consists of an early G1 phase before the restriction point, when RPCs are not
827 committed to reenter cell cycle and a late G1 phase closer to S phase when RPCs are
828 committed to reenter cell cycle (Johnson and Skotheim 2013). Thus, it is possible that in
829 the early G1 phase the RPCs can exit cell cycle to differentiate, while in the late G1
830 phase RPCs are committed to cell cycle progression. Therefore, we hypothesized that
831 GLE shortens both the early G1 phase length to accelerate developmental commitment
832 decisions of RPCs to undergo cell cycle exit and cell fate specification of RPCs, and
833 shortens the late G1 phase to accelerate developmental commitment decisions of RPCs
834 to undergo cell cycle progression. We took advantage of the known interkinetic
835 movement and localization of RPCs in different phases of cell cycle, whereby early G1
836 phase RPCs are likely to be near M phase cells in SVZ and ONBL, while late G1 phase
837 RPCs are likely to be near S phase cells in the INBL (Baye and Link 2007). In support of
838 our hypothesis, in GLE relative to control we found that SVZ and ONBL localized p16
839 INK4a and p19 INK4d were downregulated, consistent with shortening of cell cycle in
840 early G1 phase RPCs, while INBL localized CYCLINs (CYCLIN D1, A2, potentially E2)
841 were upregulated, consistent with shortening of cell cycle in late G1 phase RPCs. Other
842 studies have shown that accumulation of CDK inhibitors in developmental neurogenesis
843 slows down G1 phase length (Hindley and Philpott 2012), while CYCLIN D1 accelerates
844 G1 phase length in retina (Das et al. 2009) also support this molecular mechanism of
845 GLE mediated cell cycle length control.

846 As noted earlier, we found that GLE shortens cell cycle and increases number of
847 RPCs undergoing cell cycle reentry, cell cycle exit and cell fate specification, without
848 changing the ratio of RPCs undergoing cell cycle reentry, cell cycle exit and cell fate
849 specification. Thus, in GLE relative to control RPCs are required to quickly make
850 decisions of cell cycle reentry, cell cycle exit and cell fate specification such that the
851 number increases but the ratio of RPCs undergoing cell cycle reentry, cell cycle exit and
852 cell fate specification does not change. We found the molecular mechanism by which

853 GLE coordinates this phenotype by Affymetrix gene expression array and validation of
854 differentially expressed target genes in GLE relative to control retinas. GLE
855 simultaneously upregulated molecular regulators of RPC cell cycle reentry (NOTCH1-
856 HES1 pathway), cell cycle progression (Cdks, Notch1-Hes1 activity, Cdk4
857 phosphorylation, Rb phosphorylation), cell cycle exit (p27 Cdkn1b) and neuronal cell
858 fate specification (Otx2, Mash1, Hes6) of RPCs, without changing their spatiotemporal
859 expression pattern. It is still unclear if these diverse molecular changes in regulators of
860 cell cycle progression, cell cycle exit and neuronal cell fate regulation are an indirect
861 effect of GLE resulting from shortening of G1 phase length by GLE mediated altered
862 expression of Cyclins and INKs, or a direct effect of GLE. Moreover, it is possible that
863 these diverse molecular regulators of cell cycle progression, cell cycle exit and neuronal
864 cell fate regulation, also play a role in regulation of cell cycle length at early and late G1
865 phase. Evidence in support of a direct cell cycle length independent mechanism for
866 regulation of cell cycle progression, cell cycle exit and neuronal cell fate by GLE is
867 supported by the finding that GLE does not change glial cell fate specification factors
868 (STAT3, p-STAT3) or number of Muller glial cells. If the GLE phenotype was only a
869 result of regulation of cell cycle length, then all cell fates neuronal and glial would be
870 increased, but in GLE we only see a specific increase in neuronal cell fate factors
871 (OTX2, MASH1). Furthermore, we show that GLE increases the occupancy of c-JUN, a
872 known direct interactor of Pb (Ramesh et al. 1999), on active promoters of Cyclin D1
873 and Mash1 GLE dependent genes that regulate cell cycle length/progression and
874 neuronal cell fate specification. These findings document the molecular mechanisms by
875 which GLE specifically and directly regulates, G1 phase cell cycle length, cell cycle
876 reentry, cell cycle progression, cell cycle exit and neuronal cell fate specification, but not
877 glial cell fate specification (Fig. 7g,h).

878 Our findings in RPCs point to a novel effect of lead on neural progenitor cells of
879 cell cycle acceleration. This contrasts with other studies that have shown that lead
880 exposure slows down and impairs proliferation of neural progenitor cells in the
881 hippocampus (Gilbert et al. 2005; Schneider et al. 2005; Verina et al. 2007). One
882 possible reason for the difference could be because there are tissue specific differences
883 between retina and hippocampus, which results in different handling of lead. Another,
884 explanation for the difference is that in the GLE model the lead dose is low or moderate,
885 while the other hippocampus lead exposure models utilize a higher and chronic dose of
886 lead. Lymphocytes are not progenitor cells, but can proliferate to produce T-cells and B-
887 cells for immune response of the body. In lymphocytes, lead had a stimulatory effect on
888 proliferation at low dose, which again shows the importance of lead exposure level in
889 determining the phenotypic outcome of lead exposure (Razani-Boroujerdi et al. 1999).
890 Taken together these results suggest that the effect of lead on proliferation depends on
891 the context of tissue, timing and level of exposure.

892 Given the context dependent effect of lead on proliferation of progenitor cells, it
893 maybe expected that at the molecular level the target genes of lead will be context
894 dependent. In the context of GLE, we found upregulation of positive regulators of cell
895 cycle (NOTCH1-HES1, and CYCLINs), cell cycle exit (p27 KIP1) and rod photoreceptor,
896 and bipolar cell differentiation (OTX2, MASH1 and HES6), while GLE downregulated
897 cell cycle inhibitors (p16 INK4a and p19 INK4d). A recent study showed that in the
898 context of higher lead exposure (1mM lead acetate trihydrate) in culture media neural

899 stem cells induces oxidative stress response gene Nrf2 (Wagner et al. 2017) consistent
900 with impaired proliferation, while in lymphocytes low levels of lead activated proliferation
901 by enhancing cell-cell contact and IP3 signaling (Razani-Boroujerdi et al. 1999). Low
902 levels of lead exposure induced hepatocyte proliferation in liver by TNF α signaling
903 (Columbano et al. 1983; Kubo et al. 1996). These results demonstrate that similar to the
904 phenotypic effect of lead on progenitor cells, the molecular pathways induced by lead
905 exposure is context dependent.

906 Calcium signaling is a well-known context dependent regulator of cell cycle and
907 differentiation of progenitor cells (Carafoli 2002; Ducibella et al. 2006; Orrenius et al.
908 2003). Several studies have shown that lead can “hijack” calcium signaling pathways to
909 induce toxic effects (Fox and Katz 1992; He et al. 2000; Simons 1993). One study
910 showed that lead mediated proliferation effects in smooth muscle is rescued by calcium
911 ionophore A23187, which suggests cross-talk between lead and calcium signaling
912 pathways (Fujiwara et al. 1995). More research is needed to investigate the extend of
913 overlap between lead and calcium signaling, which could help understand how lead
914 plays a wide variety of context dependent roles in progenitor cells. The list of
915 differentially expressed GLE induced genes we have described will provide a foundation
916 for the development of new biomarkers of low dose lead toxicity (Fox et al. 2010).
917 Studies have shown that human and rodent models are in good agreement in toxicology
918 studies (Culbreth et al. 2012), which supports that our GLE results in mouse model
919 should be translatable to human GLE. We also propose that for in vitro or in vivo heavy
920 metal screening assays progenitor cells can be a great tool as a wide-variety of
921 phenotypes and molecular mechanisms that range from cell cycle to cell death can be
922 assayed using progenitor cells. Moreover, in studies of lead and other heavy metals,
923 especial care should be taken on the dose, target cell type, time and duration of
924 exposure.

925

926 **FIGURE LEGENDS**

927

928 **Figure 1: Developmental pattern of cell cycle reentry, cell cycle exit and cell fate**
 929 **specification and cell cycle phase lengths in RPCs. GLE induced accelerated cell**
 930 **cycle, increased cell cycle entry, cell cycle exit and cell fate specification.** a)
 931 Gestational lead exposure model of mice. Female mice were exposed to lead through
 932 their drinking water for 14 days prior to conception to establish a steady-state blood lead
 933 level before mating. After mating, dams were exposed to lead throughout gestation and
 934 exposure was continued from birth PN0 until PN10. This GLE model ensures that mice
 935 were exposed to lead for a period equivalent to the duration of human gestation. b-j) In
 936 mice that were injected with a single pulse of 24 hours before sacrificing for
 937 immunohistochemistry and graphical analysis of BrdU with MCM6 (b) and OTX2 (g)
 938 revealed increased number of RPCs BrdU+ (c), increased MCM6+BrdU+ cell cycle entry
 939 (d), increased MCM6-BrdU+ cell cycle exit (e), increased OTX2+BrdU+ neuronal cell fate
 940 specification in RPCs (i) and increased postmitotic neurons OTX2+ (h) in GLE. d,f)
 941 Number and ratio of RPCs reentering cell cycle decreased with age in control and GLE
 942 retinas. e,f,i,j) Number of RPCs exiting cell cycle and undergoing neuronal cell fate
 943 specification peaked at PN4 and then decreased with age, while the ratios decreased
 944 steadily with age in control and GLE retinas. Plots and graphical analysis of the labeling
 945 index vs survival time obtained from cumulative BdrU labeling and Draq5 staining reveals
 946 the total cell cycle length (Tc) as 32 hrs in control and 24 hrs in GLE, S phase length (Ts)
 947 11.5 hrs and growth fraction (GF) as 0.55 in both. The composite results from these cell
 948 cycle experiments enabled the determination of cell cycle entry phase length [cell cycle
 949 entry = $T_c - T_s - (G_2+M)$] and revealed that GLE selectively decreased the cell cycle
 950 entry phase of the cell cycle. For all quantifications data are plotted as the mean±sem,
 951 *p<0.05 and scale bar = 40 µm. Also see Supplementary Figure 1.

952

953 **Figure 2: Effect of GLE on postnatal retina gene expression using the Affymetrix**
 954 **platform.** a) Bar graph shows number of significant differentially expressed genes and
 955 Gene Ontology (GO) of these genes between GLE and control retinas. b) Heatmap
 956 presentations of genes from GOs “cell cycle” and “neuronal differentiation” expression
 957 levels measured by Affymetrix probe sets among differentially expressed between GLE
 958 and control retinas. qPCR validation of candidate “cell cycle” genes. c-m) Cell cycle
 959 activators *CyclinD1*(c), *CyclinE1*(d), *CyclinA2*(e) and cell cycle regulators/markers
 960 *Mcm6*(f), *Cdk4*(g), *Cdk2*(h), were upregulated in GLE. *Hes1*(i) and *Hes6*(j) were
 961 upregulated in GLE. Cell cycle inhibitors *p16 Ink4a*(k) and *p19 Ink4d*(l) were
 962 downregulated in GLE. Cell cycle regulator *Rb1*(m) and cell fate specification factor *p27*
 963 *Kip1*(n) were upregulated in GLE. qPCR validation of candidate “neuronal differentiation”
 964 genes. o-q) Proneuronal rod-bipolar cell fate specification and differentiation factors,
 965 *Otx2*(o) and *Mash1*(p) were upregulated in GLE, but there was no change in *Neurod1*(q).
 966 For all quantifications data are plotted as the mean±sem, *p<0.05.

967

968 **Figure 3: Expression of CYCLINs decreases with development age and is GLE**
 969 **upregulated by GLE.** Immunolabeling of CYCLIN D1(a) and CYCLIN A2(b) are highest
 970 in S-phase RPCs in INBL and present in RPCs in SVZ of GLE and control retinas.
 971 However, in GLE retinas the number of these cyclin colabeled RPCs was increased. c-l)

972 Quantification by western blot and densitometry show age dependent decreased
 973 expression of cell cycle proteins, CYCLIN D1(c,d), CYCLIN E2(c,e), CYCLIN A2(c,f),
 974 MCM6(c,g), RB1(c,h), pRB1(c,i), CDK4(c,j), CDK2(c,k) and pCDK2(c,l) consistent with
 975 completion of development in control and GLE retinas. In GLE expression of all these cell
 976 cycle proteins, was upregulated. These results are consistent with increased cell cycle
 977 progression and cell cycle entry in GLE relative to control. For all quantifications data are
 978 plotted as the mean \pm sem, *p<0.05. Scale bar = 40 μ m. Also, see Supplementary Figure
 979 2.

980

981 **Figure 4: Expression of INKs decreases with developmental age and p27 KIP1**
 982 **expression peaks at PN4. GLE downregulates expression of INKs and upregulates**
 983 **expression of p27 KIP1.** Immunolabeling of cell cycle inhibitors p16 INK4a(a) and p19
 984 INK4d(b), and cell cycle exit regulator p27 KIP1(c) are highest in RPCs in SVZ of GLE
 985 and control retinas. However, in GLE retinas the number of these cell cycle inhibitor p16
 986 INK4a and p19 INK4d colabeled RPCs was decreased, while p27 KIP1 cell cycle exit
 987 regulator colabeled RPCs was increased. d-g) Quantification by western blot and
 988 densitometry show age dependent decreased expression of cell cycle inhibitor proteins,
 989 p16 INK4a(d,e) and p19 INK4d(d,f) consistent with completion of development, and peak
 990 of cell cycle exit regulator p27 KIP1(d,g) consistent with peak of cell cycle exit/cell fate
 991 specification in control and GLE retinas. In GLE expression of p16 INK4a and p19 INK4d
 992 was downregulated, while that of p27 KIP1 was upregulated. These results are consistent
 993 with increased cell cycle progression, cell cycle entry and cell cycle exit in GLE relative
 994 to control. For all quantifications data are plotted as the mean \pm sem, *p<0.05. Scale bar =
 995 40 μ m. Also, see Supplementary Figure 2.

996

997 **Figure 5: Expression of MASH1 increases with developmental age, while OTX2,**
 998 **NEUROD1 and HES6 peak at PN4. GLE upregulates expression of MASH1, OTX2**
 999 **and HES6.** Immunolabeling of OTX2(a) and MASH1(b) are highest in the SVZ RPCs where
 1000 cell cycle exit and cell fate specification occurs in control and GLE retinas. However, in
 1001 GLE retinas the number of OTX2 and MASH1 colabeled RPCs was increased. c-g)
 1002 Quantification by western blot and densitometry shows peak expression at PN4 of cell
 1003 fate specification factors OTX2(c,d), NEUROD1(c,e), HES6(c,f) and MASH1(c,g)
 1004 consistent with peak of cell cycle exit and rod-bipolar cell fate specification in control and
 1005 GLE retinas. In GLE expression of all OTX2, HES6 and MASH1 were upregulated, while
 1006 NEUROD1 did not change. For all quantifications data are plotted as the mean \pm sem,
 1007 *p<0.05. Scale bar = 40 μ m. Also see Supplementary Figure 3.

1008

1009 **Figure 6: Expression and activation of NOTCH1-HES1 pathway decreases with**
 1010 **developmental age, while STAT3 activation peaks and sustains after PN4. GLE**
 1011 **differentially regulates Notch1-Hes1 pathway to maintain RPCs early on and make**
 1012 **RPCs permissive for differentiation at PN6.** a) Immunolabeling of NOTCH1, HES1 and
 1013 Ki67 shows that early on at PN2 there is more colabeling of RPCs with HES1 and more
 1014 labeling of Notch in SVZ in control and GLE retinas consistent with role of NOTCH1-HES1
 1015 pathway in maintenance or cell cycle entry of RPCs. This colabeling of HES1 RPC
 1016 colabeling was increased in GLE. At PN6 HES1 and Ki67 were localized in inner retina
 1017 but did not colabel (HES1 is known to express in Müller glial cells at this age). b-g)

1018 Quantification by western blot and densitometry show age dependent decreased
 1019 expression of NOTCH1(b,c), NOTCH1 activation (NOTCH1-ICD)(b,d) and HES1(b,e)
 1020 consistent with completion of development in control and GLE retinas. Müller glial cell
 1021 fate specification factors STAT3(f) and pSTAT3(g) peaked and stayed up PN6 onwards
 1022 in control and GLE retinas, overlapping with known Muller glial cell specification timing.
 1023 In GLE expression of all NOTCH1-HES1, was upregulated upto PN4 supporting
 1024 increased cell cycle entry of RPCs and went down PN6 onwards making it permissive to
 1025 rod-bipolar cell fate specification. Interestingly, STAT3 and pSTAT3 did not change in
 1026 GLE consistent with no change in Muller glial cell number in GLE. For all quantifications
 1027 data are plotted as the mean \pm sem, *p<0.05. Scale bar = 40 μ m. Also see Supplementary
 1028 Figure 3.

1029

1030 **Figure 7: Developmental age and GLE dependent transcription factor occupancy**
 1031 **on CyclinD1 and Mash1 promoters. Summary model.** ChIP-qPCR was performed to
 1032 evaluate the binding and activity of CyclinD1 and Mash1 promoter. a-c) In control and
 1033 GLE retinas, RNA POL2 and E2F1 exhibited significant age-dependent decreases in
 1034 relative binding to CyclinD1-promoter from PN2-PN4 to PN6. GLE increases the relative
 1035 binding of RNA POL2, E2F1 and C-JUN at PN2 and PN4, while N-MYC binding increases
 1036 in GLE at PN4 only. d-f) In control and GLE PN2-PN6 retinas, RNA POL2 and C-JUN
 1037 exhibited significant binding peaks to Mash1 at PN4, while E2F1 exhibited significantly
 1038 high binding to Mash1 at PN2 and PN4. Furthermore, in GLE retinas the relative binding
 1039 of RNA POL2 and C-JUN significantly increased from PN2 to PN4, whereas E2F1 binding
 1040 significantly increased at PN2 and PN4. g) Summary of spatial distribution of cell cycle
 1041 regulators (CYCLINs, INKs), cell cycle exit regulator p27 KIP1, neuronal cell fate
 1042 specification factors (MASH1, OTX2) and NOTCH1-HES1 RPC self-renewal pathway in
 1043 SVZ, ONBL and INBL of developing postnatal retina. Intensity of color is directly
 1044 proportional to expression level, and checkered pattern specifically indicates switched
 1045 expression pattern at PN6. h) Summary model for cell cycle reentry, cell cycle exit and
 1046 neuronal cell fate specification. CYCLINs and INKs regulate CDK activity and RB
 1047 phosphorylation, which determines number of RPCs cell cycle entry in retina. GLE
 1048 upregulates CYCLINs and downregulates INKs to increase cell cycle entry, cell cycle
 1049 progression and shorten length of G1 phase of cell cycle. NOTCH1-HES1 pathway, p27
 1050 and MASH1, regulate the number of RPCs that exit cell cycle to acquire rod and bipolar
 1051 cell fate. Moreover, these results suggest that GLE alters NOTCH1-HES1 pathway and
 1052 G00sp Otx2, Hes6 and Mash1 and cell cycle exit factor p27 to increase neurogenesis of
 1053 rod and bipolar cells. For all quantifications data are plotted as the mean \pm sem, *p<0.05.

1054

1055

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1359

1360 **Access to Codes:**

1361 This work does not contain any codes. Please cite this paper if you use this paper.

1362 Thank you.

1363

1 **FIGURE LEGEND**

2 **Supplementary Figure 1, related to Figure 1: Developmental pattern of cell**
3 **cycle reentry, cell cycle exit and cell fate specification and cell cycle phase**
4 **lengths in RPCs. GLE induced accelerated cell cycle, increased cell cycle**
5 **entry, cell cycle exit and cell fate specification.** a,b) In mice that were injected
6 with a single pulse of 24 hours before sacrificing for immunohistochemistry
7 analysis of BrdU with MCM6 (a) and OTX2 (b) revealed increased number of
8 MCM6+BrdU+ RPCs reentering cell cycle, increased number of MCM6-BrdU+
9 RPCs exiting cell cycle, and increased number of BrdU+OTX2+ RPCs becoming
10 postmitotic OTX2+ neurons in GLE. c) In GLE MCM6 and BrdU labeling, 45 min
11 BrdU pulse before sacrificing, reveals accelerated cell cycle or higher
12 proliferation index of RPCs. d) GLE did not alter the G2+M phase of cell cycle
13 calculated as 4.5 hrs from the fraction of PH3⁺ and MCM6⁺ colabeled cells
14 counted at 2.5, 3.5 and 4.5 hours following a BrdU injection and the time to reach
15 a mitotic fraction of 1.0 was determined. For all quantifications data are plotted as
16 the mean±sem, *p<0.05 and scale bar = 40 μm.

17 **Supplementary Figure 2, related to Figure 3 and Figure 4: Expression of**
18 **CYCLINS and INKs decreases with developmental age and p27 KIP1**
19 **expression peaks at PN4. GLE downregulates expression of INKs and**
20 **upregulates expression of CYCLINS and p27 KIP1.** Immunolabeling of
21 CYCLIN D1(a) and CYCLIN A2(b) are highest in S-phase RPCs in inner retina
22 and also present in RPCs in SVZ of GLE and control retinas. However, in GLE
23 retinas the number of these cyclin colabeled RPCs was increased.

24 Immunolabeling of cell cycle inhibitors p16 INK4a(c) and p19INK4d(d), and cell
25 cycle exit regulator p27 KIP1(e) are highest in RPCs in SVZ of GLE and control
26 retinas. However, in GLE retinas the number of these cell cycle inhibitor
27 p16INK4a and p19INK4d colabeled RPCs was decreased, while p27 KIP1 cell
28 cycle exit regulator colabeled RPCs was increased. f,g) Quantification by
29 western blot and densitometry show age dependent decreased expression of cell
30 cycle proteins, p107(f) and E2F1(g) consistent with completion of development in
31 control and GLE retinas. In GLE expression of p107 and E2F1 cell cycle proteins
32 were unchanged. All quantifications data are plotted as the mean \pm sem, *p<0.05.
33 Scale bar = 40 μ m.

34 **Supplementary Figure 3, related to Figure 5 and Figure 6: Expression of**
35 **NOTCH1, HES1, OTX2, and MASH1 neuronal cell fate specification factors**
36 **during development. GLE increases rod and bipolar cell fate specification**
37 **factors and alters NOTCH1-HES1 pathway.** Immunolabeling of OTX2(a) and
38 MASH1(b) are highest in the SVZ RPCs where cell cycle exit and cell fate
39 specification occurs in control and GLE retinas. However, in GLE retinas the
40 number of OTX2 and MASH1 colabeled RPCs was increased. At PN8 OTX2 was
41 localized in inner retina or INBL. c) Immunolabeling of NOTCH1, HES1 and Ki67
42 shows that early on at PN4 there is more colabeling of RPCs with Hes1 and
43 more labeling of Notch in SVZ in control and GLE retinas consistent with role of
44 NOTCH1-HES1 in maintenance of RPCs. This colabeling of HES1 RPC
45 colabeling was increased in GLE. At PN8 HES1 was localized in inner retina or

46 INBL (HES1 is known to express in Muller glial cells at this age). For all
47 quantifications data are plotted as the mean \pm sem, * p <0.05. Scale bar = 40 μ m.

Supplement Table 1: Cell Specific Primary Antibodies and Dye Used for Immunohistochemistry (IHC) and Western Blot (WB)

| Primary Antigen | Source and Catalog No. | Target | Host | Dilution | IHC, WB |
|--------------------|---------------------------------|---|--------|--------------|----------|
| BrdU | BD Biosciences 347580 | Labels RPCs in S phase | Mouse | 1:100 | IHC |
| MCM6 | Santa Cruz sc-9843 | RPCs | Goat | 1:100, 1:200 | IHC, WB |
| DRAQ5 | Cell signaling technology 4084S | Nucleus | | 1:250 | IHC |
| CCND1 or Cyclin D1 | Santa Cruz sc-450 | RPCs | Mouse | 1:100, 1:200 | IHC, WB |
| CCNE2 or Cyclin E2 | Santa Cruz sc-22777 | G1/S phase | Rabbit | 1:500 | WB |
| CCNA2 or Cyclin A2 | Invitrogen 33-4900 | RPCs in S phase | Mouse | 1:100, 1:250 | IHC, WB |
| P16Ink4a or CDKN2A | Santa Cruz sc-1661 | Early postmitotic cells; few in RPCs | Mouse | 1:50, 1:500 | IHC, WB |
| P19Ink4d or CDKN2D | Santa Cruz sc-1063 | Perinuclear labeling in early postmitotic cells and few in RPCs | Rabbit | 1:50, 1:200 | IHC, WB |
| P27Kip1 or CDKN1B | Cell Signaling Technology 2552 | Early postmitotic cells; few in RPCs | Rabbit | 1:1000 | WB |
| P27Kip1 or CDKN1B | BD Biosciences 610241 | Early postmitotic cells; few in RPCs.; also Muller cells. | Mouse | 1:100 | IHC |
| CDK4 | Santa Cruz sc-260 | G1 phase cyclin dependent kinase | Rabbit | 1:50 | WB |
| CDK2 | Santa Cruz sc-6248 | S and G2 phase cyclin dependent kinase | Mouse | 1:50 | WB |
| p-CDK2 (Thr 160) | Santa Cruz sc-101656 | Activated Cdk2 | Rabbit | 1:50 | WB |
| RB1 | BD Biosciences 554136 | Retinoblastoma protein | Mouse | 1:250 | WB |
| p-RB1 (Ser 780) | Cell Signaling Technology 9307S | Hyperphosphorylated-RB | Rabbit | 1:1000 | WB |
| P107 | Santa Cruz sc-318 | P107 from RB family | Rabbit | 1:200 | WB |
| E2F1 | Santa Cruz sc-193X | E2F1 transcription factor | Rabbit | 1:1000 | ChIP-PCR |
| C-JUN | Santa Cruz sc-45X | C-JUN transcription factor | Rabbit | 1:1000 | ChIP |
| POL II (N-20) | Santa Cruz sc-899 | RNA Polymerase | Rabbit | 1:1000 | ChIP |
| PH3 (Ser10) | Upstate 06-570 | Labels RPCs in M phase | Rabbit | 1:40 | IHC |

| | | | | | |
|-----------------|---------------------|-----------------------------------|--------|-----------------|-----------|
| Otx2 | Chemicon AB9566 | OTX2 homeodomain protein | Rabbit | 1:100 1:200 | IHC WB |
| Ki67 | BD 550609 | Retinal progenitor cells | Mouse | 1:100 | IHC |
| MASH1 | Chemicon ab15582 | RPC, and Differentiating neurons | Rabbit | 1:100 1:1000 | IHC WB |
| HES6 | Gift | bHLH transcription factor HES6 | Rabbit | 1:100 | WB |
| NEUROD1 | Chemicon AB5686 | bHLH transcription factor NEUROD1 | Rabbit | 1:500 | WB |
| STAT3 | Cell Signaling 9139 | STAT3 transcription factor | Mouse | 1:500 | WB |
| pSTAT3 (Tyr705) | Cell Signaling 9145 | Activated STAT3 | Rabbit | 1:500 | WB |
| NOTCH1 | Santa Cruz sc-6015 | NOTCH1 membrane protein | Goat | 1:100 | IHC |
| NOTCH1-ECD | Upstate 07-218 | NOTCH1 membrane protein | Rabbit | 1:1000 | WB |

| | | | | | |
|------------|-----------------|--------------------------------|--------|-----------------|-----------|
| NOTCH1-ICD | Upstate 07-220 | Internal cleaved active NOTCH1 | Rabbit | 1:1000 | WB |
| HES1 | Chemicon ab5702 | RPCs, and Muller glial cells | Rabbit | 1:100 1:1000 | IHC WB |

Supplement Table 2: RT-qPCR and ChIP-PCR Gene-Specific Forward and Reverse primers

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') | Product Size (bp) | Location intron |
|----------------|------------------------|--------------------------|-------------------|---------------------------------------|
| Cyclin D1-1 | aacaagcagaccatccgcaa | tggagggtgggttgaaatgaa | 85 | Exons 3-4 |
| Ccne2 | gccatcgactctttagaattca | tgtcatcccattccaaact | 109 | 769(1157) |
| Ccna2-2 | gtcaaccccgaaaactgg | aaggtcctaagaggagcaacc | 129 | 655(360) |
| p19-1 | gtcggcgaccggtgagt | tgtctggactccaacatca | 144 | 401 |
| P16 | cgacgggcatagcttcag | gctctgctctgggattgg | | |
| P27-1 | agtgccagggatgaggaag | tctgttctgtggccctttt | 73 | 559 bp |
| Mcm6-1 | acctgtaccacaatctctgcac | caccgcgtttacttcatca | 69 | 2353(1169) |
| Cdk4 | tcagtgtgccagagatgg | ggaaggcagagattcgctta | 88 | 992 |
| Cdk2 | ctgcatcttctgaaatgg | gatccggaagagtgtgtaat | | |
| Rb1-1 | aaagaagtctgaaggcggcaa | aatttgactccgctgggagat | 112 | Exons 24-25 |
| P107-1 | gcggcaactacagcctaga | tgcggcaagcaacatataaa | 69 | 4747 |
| E2f1-1 | tgccaagaagtccaagaatca | ctcaagccgcttccaatc | 74 | 804 |
| Rb1-pro2 | caacccgcaaaagtggaa | atccggcctccttccataat | 78 | -597/-674;chr14: 73726195-73726272 |
| CCND1-pro3 | ctagctgtcctctgtccaga | gggcttcttccctaagagg | 77 | (upstream)_248 to _172 |
| <i>Otx2</i> | ggtatggacttgcctcatcc | cgagctgtgccctagtaaatg | 91 | 992 |
| <i>Neurod1</i> | gcagaaggcaaggtgtcc | tftggtcatgttccactcc | 89 | 1497 |
| <i>Ascl1</i> | cgaatcacagatgggttct | gtggttgggagttaatagtgctg | 88 | 20494 |
| <i>Hes6</i> | acggatcaacgagagtctca | ttctctagcttggcctgcac | 72 | 84 |
| <i>Notch1</i> | ctggaccccatggacatc | aggatgactgcacacattgc | 73 | 1012 |
| <i>Hes1</i> | acaccggacaaaccaagac | cgcctcttccatgatagg | 68 | 130 |
| <i>Stat3</i> | tggcaccttgattgagag | caacgtggcatgtgactctt | 65 | 2427 |
| <i>Ascl1</i> | tgtttattcagccgggagtc | gtccttgcaactctcca | 84 | -183/-266;chr10: 86956588-86956671 |
| <i>β-actin</i> | agagaggtatctgacctgaagt | cacgcagctcattgtagaaggtgt | 105 | 262-366 |

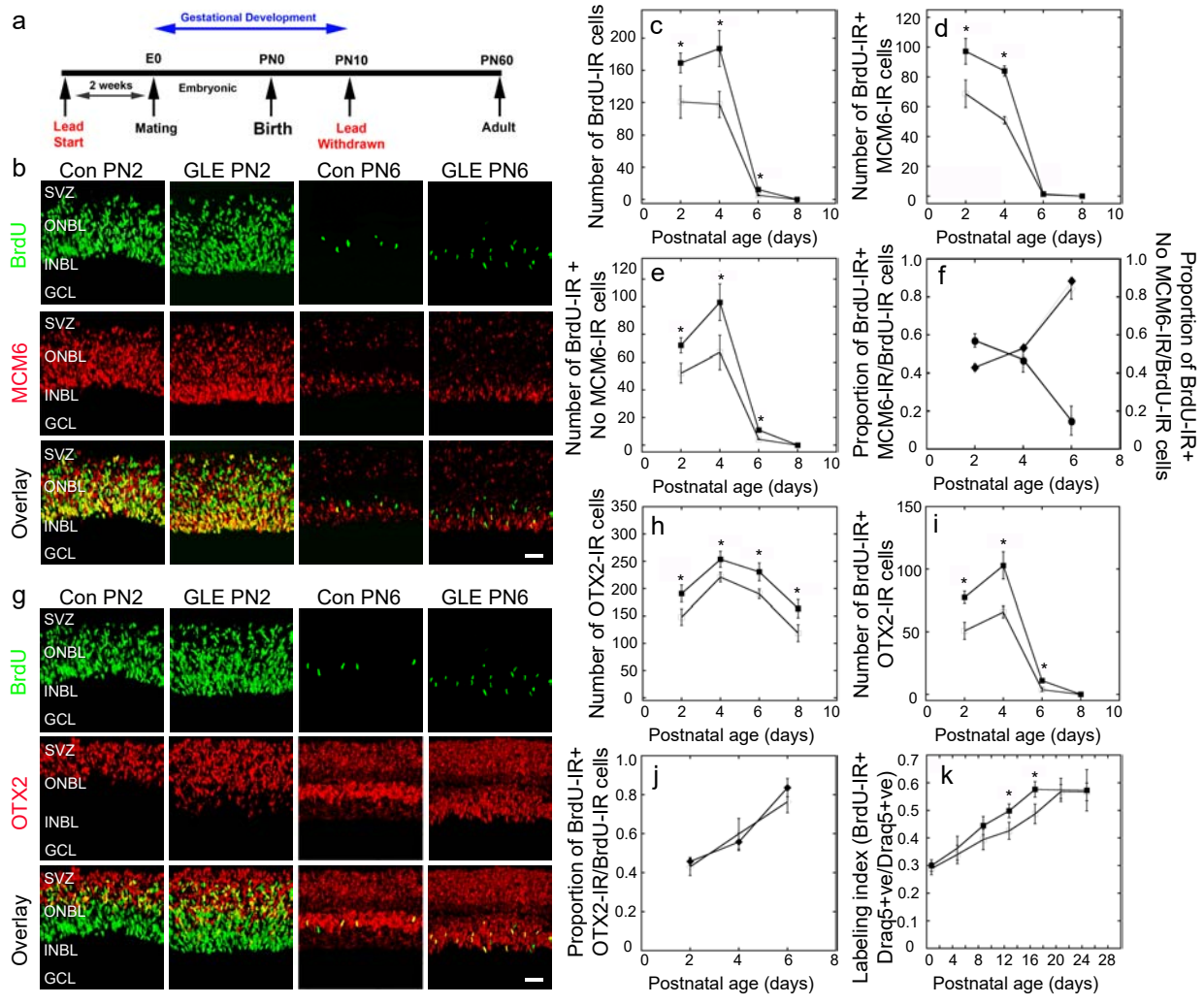


Figure 1 Mukherjee

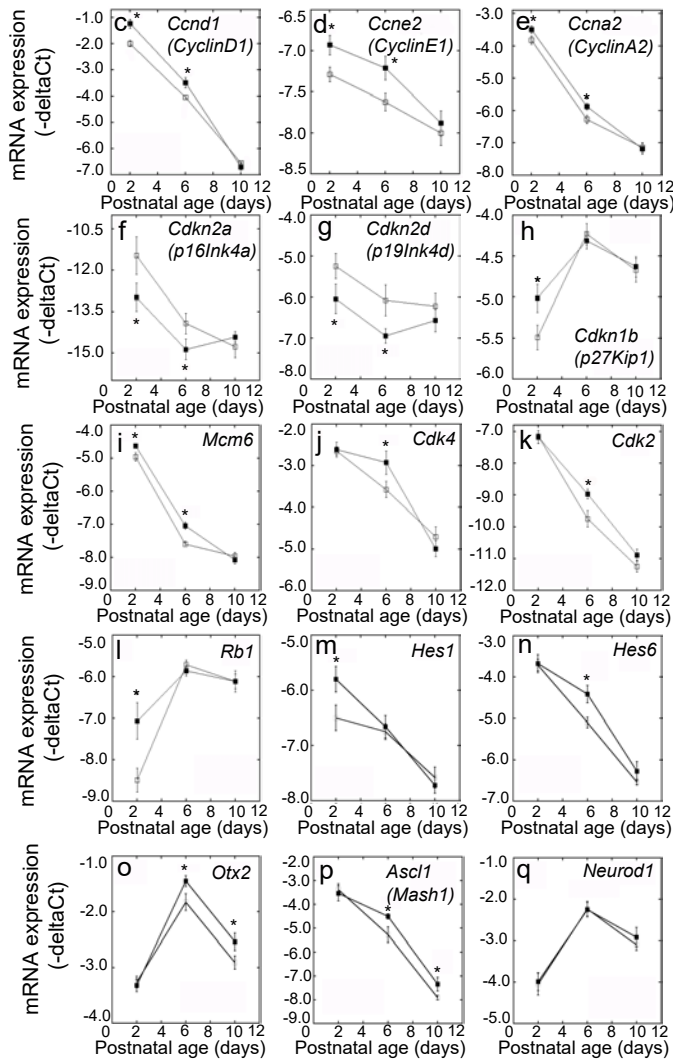
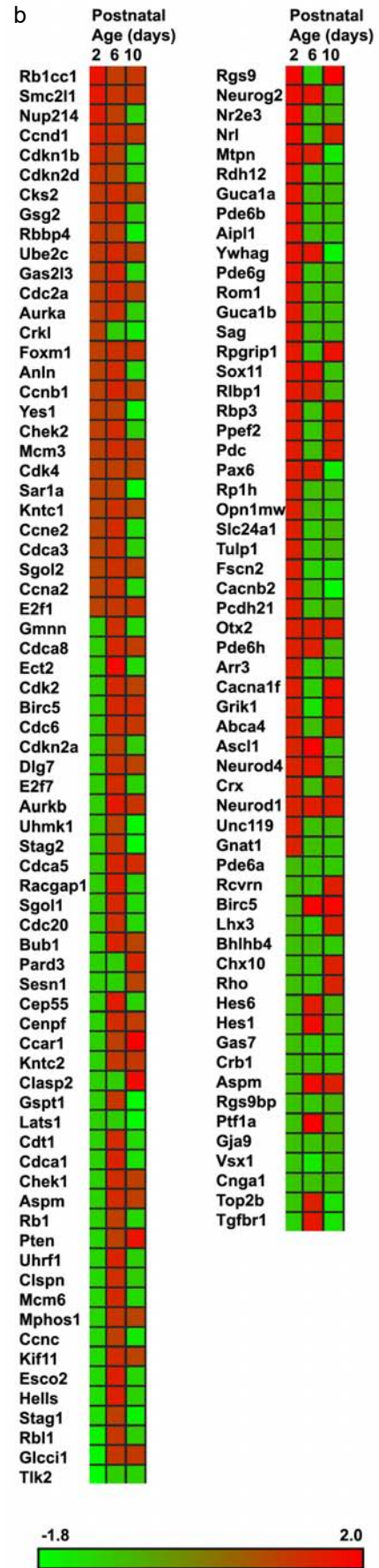
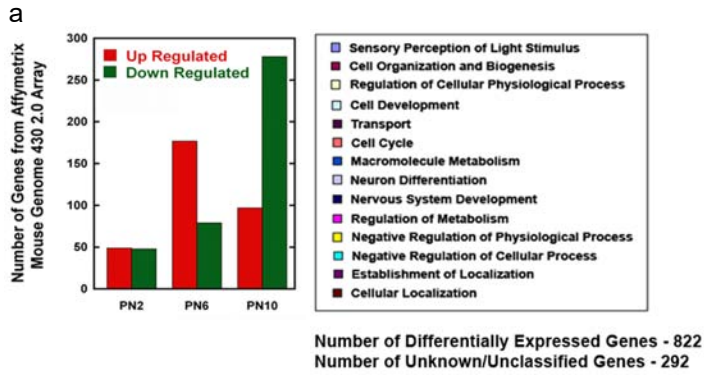


Figure 2 Mukherjee

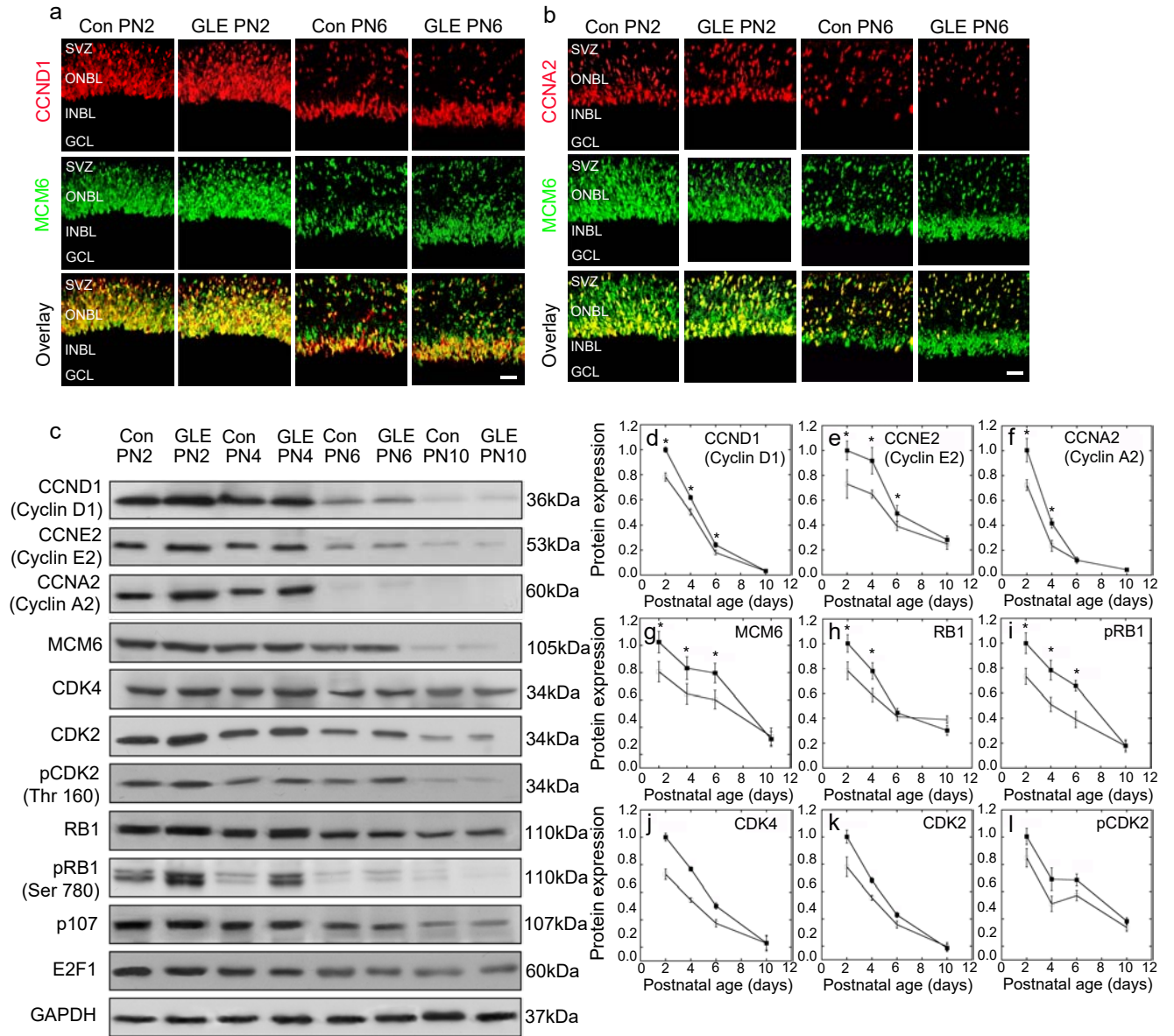


Figure 3 Mukherjee

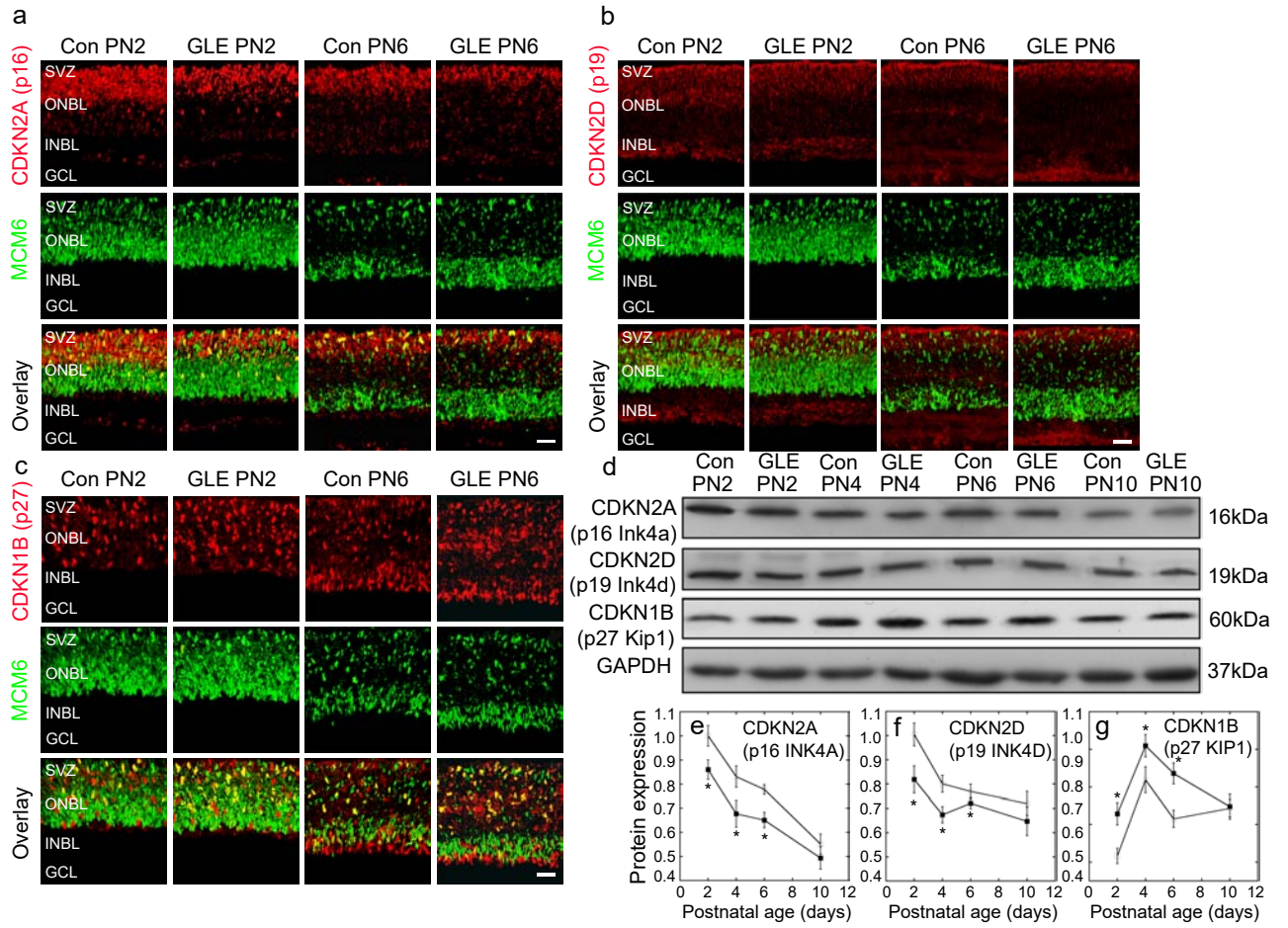


Figure 4 Mukherjee

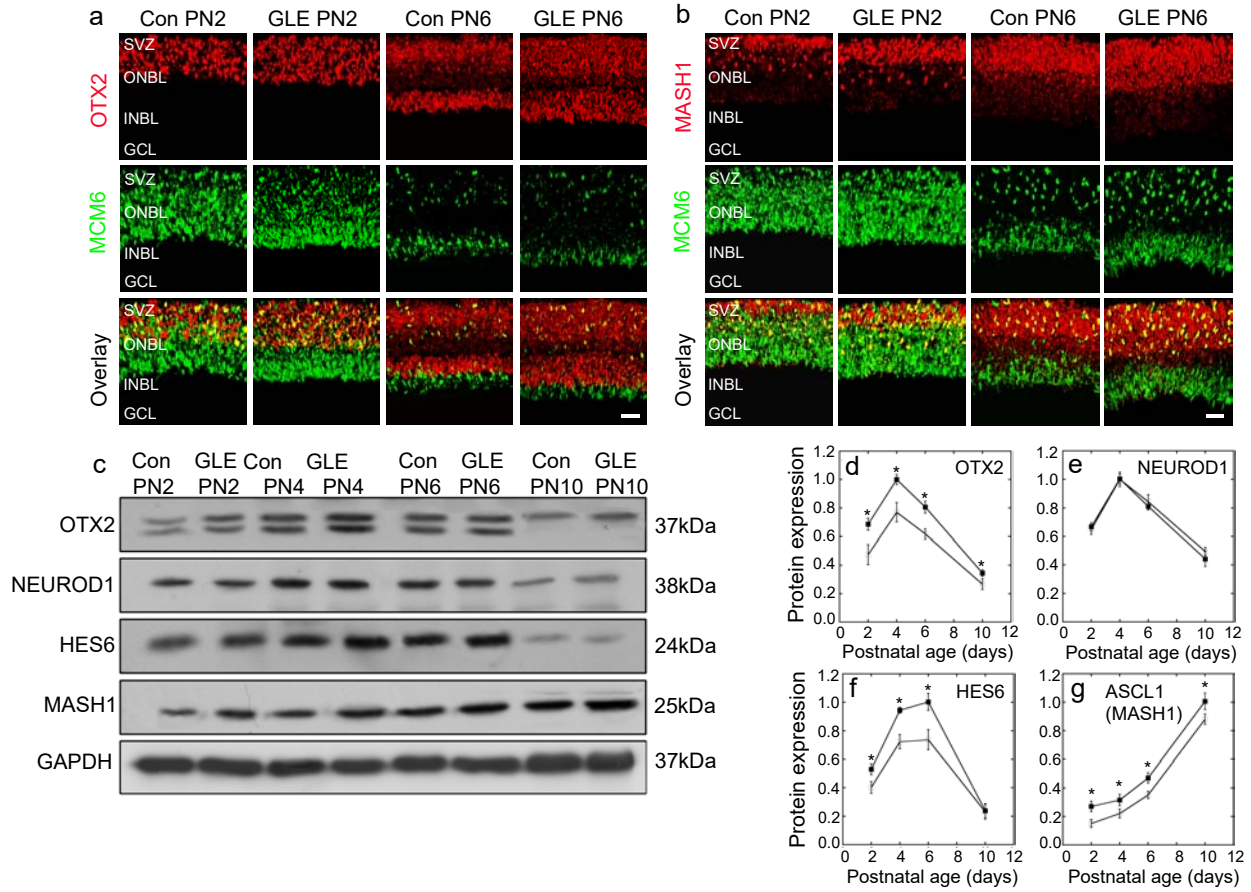


Figure 5 Mukherjee

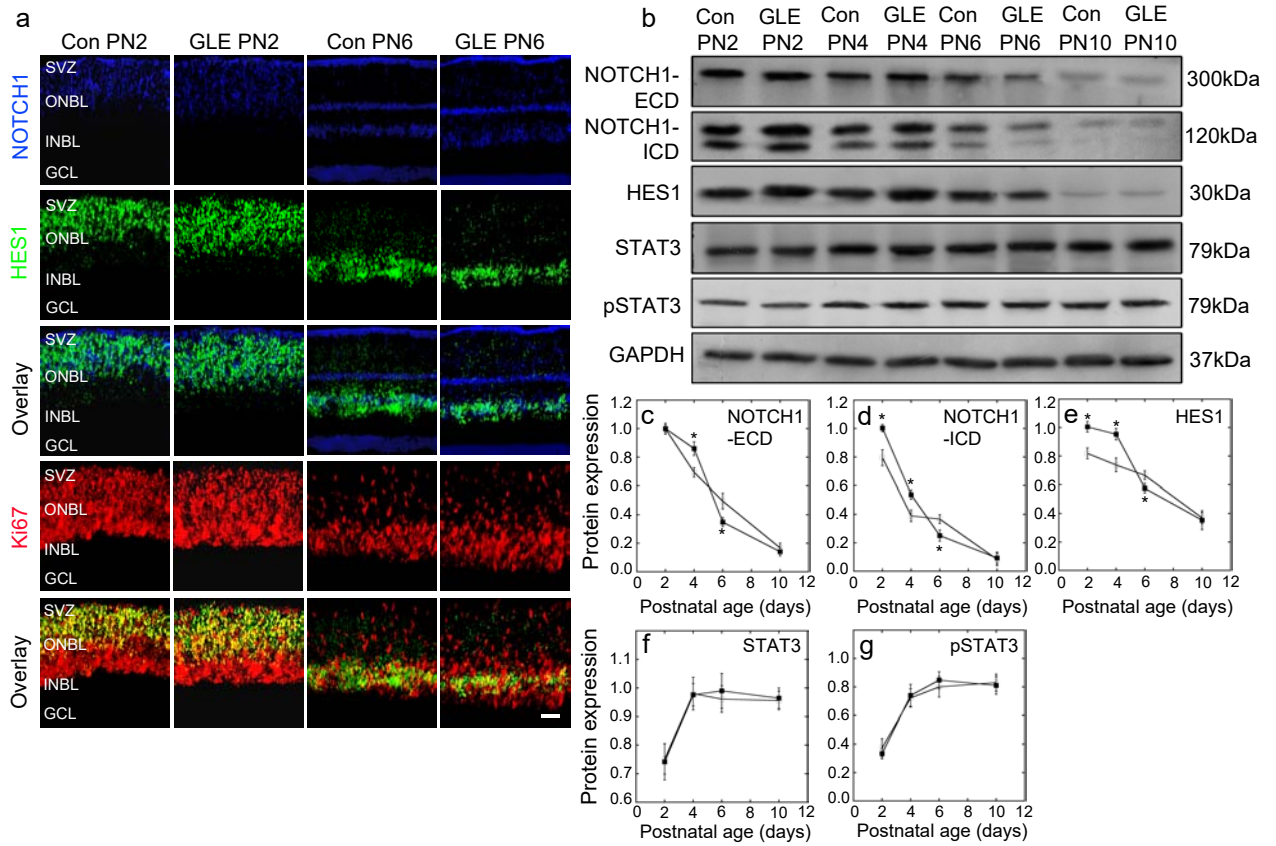


Figure 6 Mukherjee

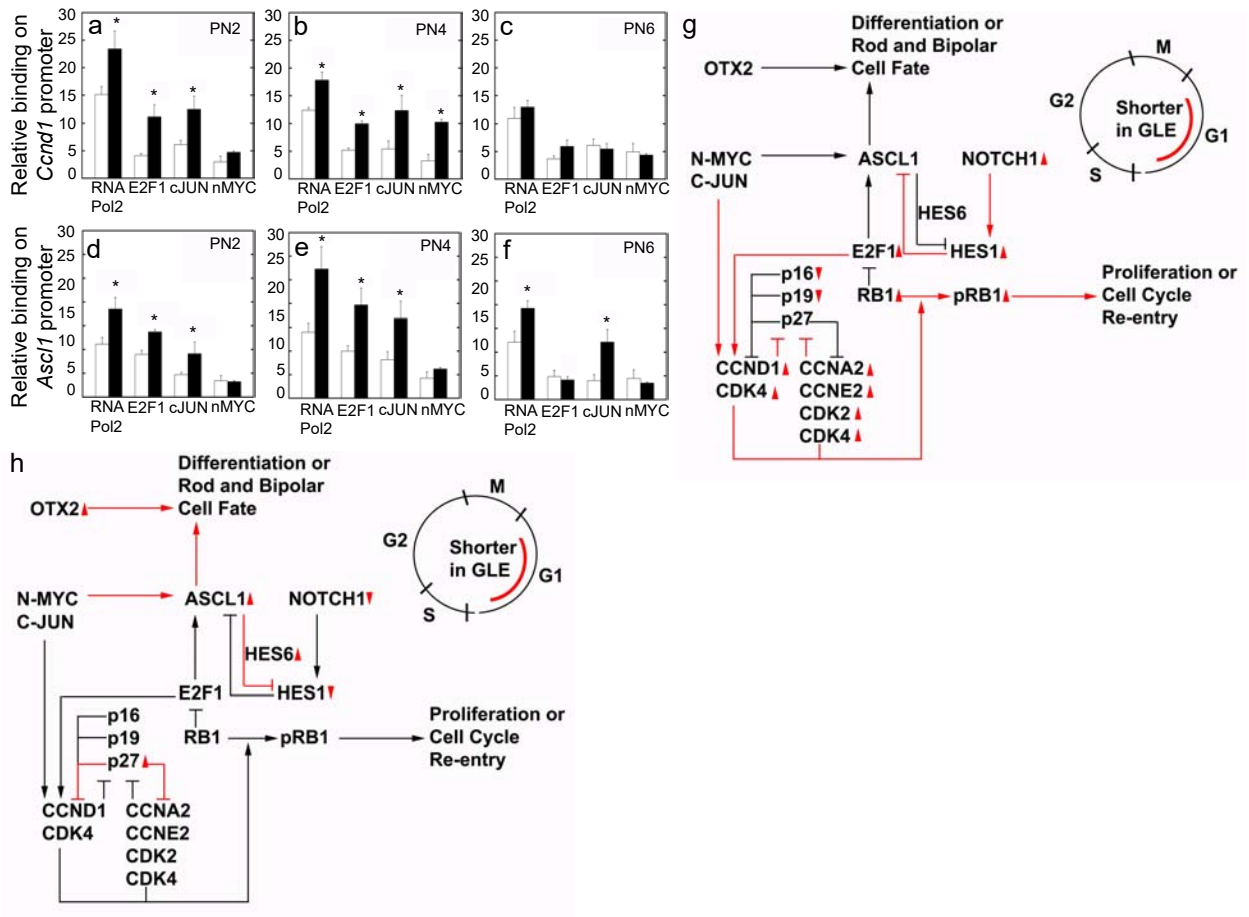
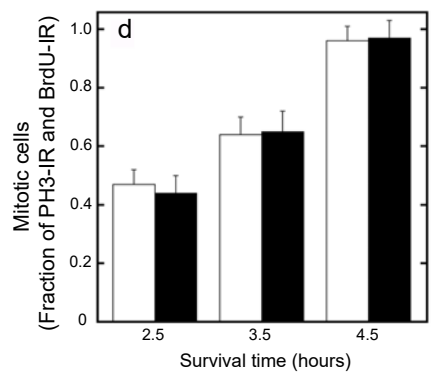
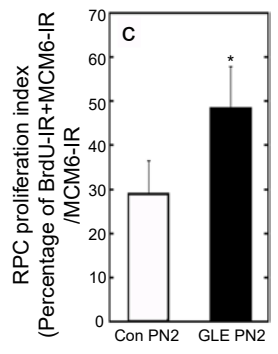
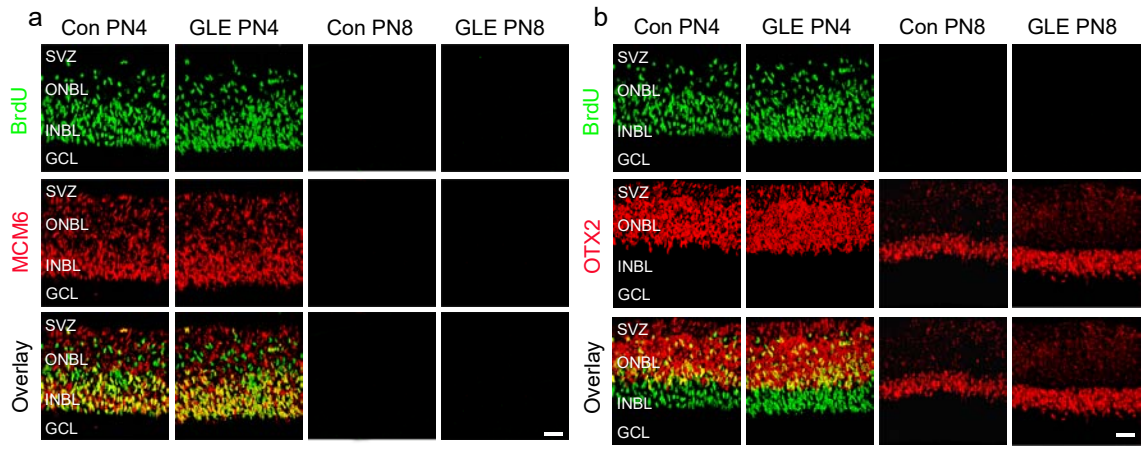
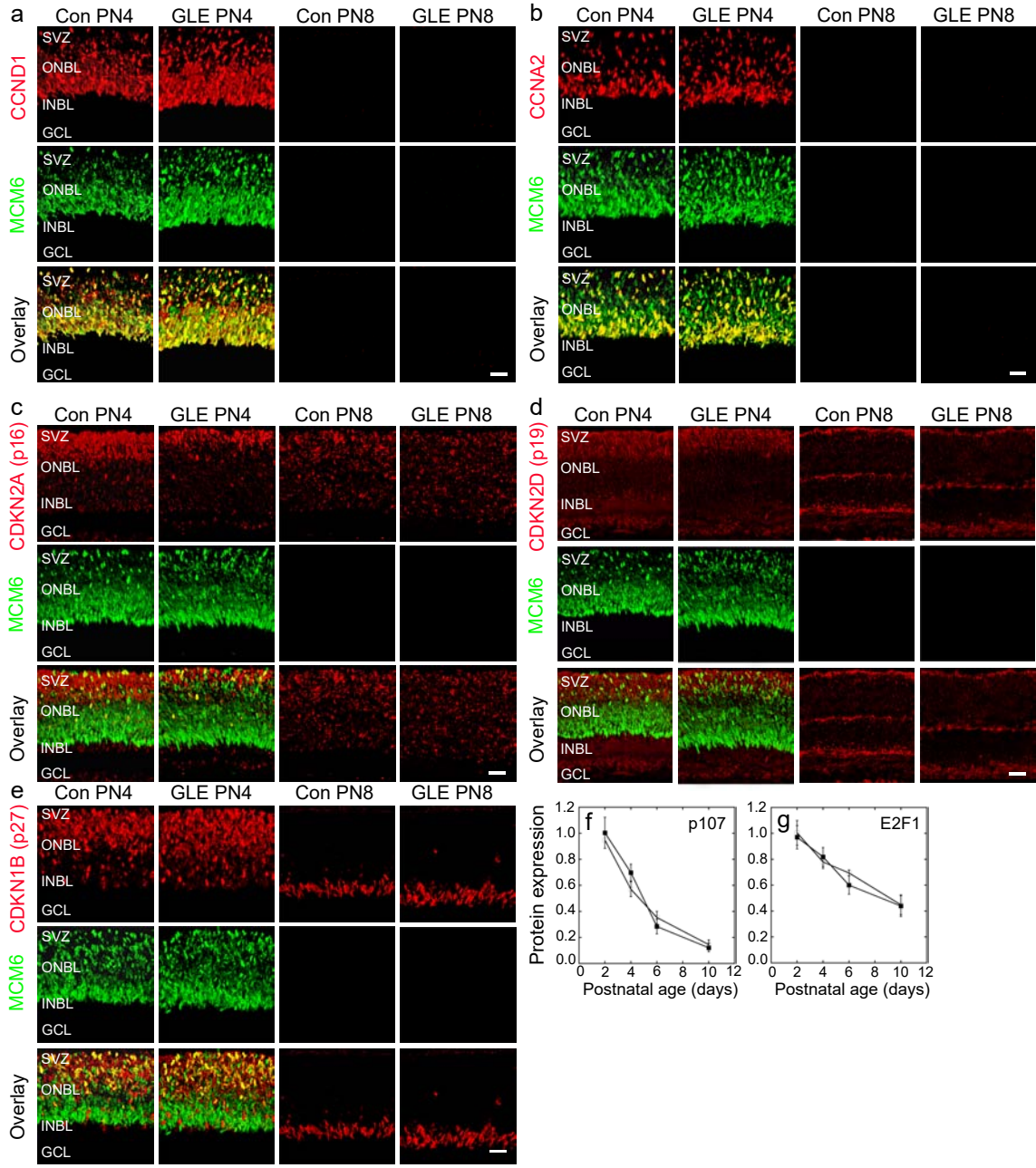


Figure 7 Mukherjee





Supplementary Figure 2 Mukherjee

