

1 Atoh7-independent specification of retinal ganglion cell 2 identity

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36 **Abstract**

37 Retinal ganglion cells (RGCs), which relay visual information from the eye to the brain,
38 are the first cell type generated during retinal neurogenesis. Loss of function of the
39 transcription factor *Atoh7*, which is expressed in multipotent early neurogenic retinal
40 progenitor cells, leads to a selective and near complete loss of RGCs. *Atoh7* has thus
41 been considered essential for conferring competence on progenitors to generate RGCs.
42 However, when apoptosis is inhibited in *Atoh7*-deficient mice by loss of function of *Bax*,
43 only a modest reduction in RGC number is observed. Single-cell RNA-Seq of
44 *Atoh7*;*Bax*-deficient retinas shows that RGC differentiation is delayed, but that RGC
45 precursors are grossly normal. *Atoh7*;*Bax*-deficient RGCs eventually mature, fire action
46 potentials, and incorporate into retinal circuitry, but exhibit severe axonal guidance
47 defects. This study reveals an essential role for *Atoh7* in RGC survival, and
48 demonstrates *Atoh7*-independent mechanisms for RGC specification.

49 **Introduction**

50 The retina has six major classes of neurons that develop from a common progenitor cell
51 pool during overlapping temporal intervals. Retinal ganglion cells (RGCs), the only
52 projection neurons from the retina to the brain, are the first retinal cell type to be
53 generated. RGC development in both zebrafish, mice, and humans has been shown to
54 require the basic helix-loop-helix transcription factor atonal homolog 7 - *Atoh7* (*Math5*)
55 (Brown et al., 2001; Ghiasvand et al., 2011; Jarman et al., 1994; Khan et al., 2011; Khor
56 et al., 2011; Macgregor et al., 2010; Prasov et al., 2012; Wang et al., 2001). *Atoh7* is
57 conserved across all vertebrate species, and distantly related to *atonal*, which specifies
58 the earliest-born neurons in *Drosophila* retina (Brown et al., 2001; Jarman et al., 1994;
59 Kanekar et al., 1997; Prasov et al., 2012). *Atoh7*-deficient mice and zebrafish lack
60 upwards of 95% of RGCs (Brown et al., 2001; Brzezinski et al., 2012; Kay et al., 2001;
61 Wang et al., 2001) and likewise lack any visible optic nerve or functional connections
62 from the retina to the brain (Brzezinski et al., 2005; Wee et al., 2002). Human mutations
63 in *ATOH7* or its cis-regulatory regions have been associated with optic nerve agenesis
64 or hypoplasia (Khan et al., 2012; Macgregor et al., 2010) and increased susceptibility to
65 glaucoma (Fan et al., 2011; Ramdas et al., 2011). *Atoh7*-deficiency also disrupts
66 development of retinal vasculature in both mice and humans, likely as an indirect result
67 of the loss of RGCs (Edwards et al., 2012).

68 In mice, *Atoh7* is expressed in neurogenic retinal progenitor cells (RPCs)
69 between E12 and P0, corresponding to the interval in which RGCs are generated
70 (Brown et al., 1998; Clark et al., 2019; Feng et al., 2010; Pacal and Bremner, 2014;
71 Prasov and Glaser, 2012; Rapaport et al., 2004; Sidman, 1960; Wang et al., 2001;
72 Young, 1985). Upon cell fate specification, *Atoh7* expression is rapidly down-regulated
73 in mouse RGC precursors (Clark et al., 2019; Miesfeld et al., 2018a) although
74 expression persists in immature human RGCs (Aparicio et al., 2017; Lu et al., 2020).
75 Genetic fate mapping indicates that *Atoh7*-expressing RPCs also give rise to other
76 early-born retinal cells, including cone photoreceptors, horizontal and amacrine cells,
77 and that generation of these cell types is increased in *Atoh7*-deficient mice (Brown et
78 al., 2001; Brzezinski et al., 2005; Hufnagel et al., 2013; Wang et al., 2001). Ectopic
79 expression of *Atoh7* alone, however, typically is not sufficient to drive RGC specification

80 (Gan et al., 1999; Hufnagel et al., 2013; Mao et al., 2013, 2008; Ohnuma et al., 2002;
81 Pan et al., 2008; Pittman et al., 2008; Prasov and Glaser, 2012; Wu et al., 2015),
82 although misexpression of *Atoh7* in Crx-expressing photoreceptor precursors was
83 sufficient to rescue development of a limited number of RGCs (Prasov and Glaser,
84 2012).

85 These findings have suggested that *Atoh7* acts in neurogenic RPCs to confer
86 competence to generate RGCs (Brzezinski et al., 2012; Mu et al., 2005; Yang et al.,
87 2003) potentially in combination with as yet unidentified factors. Recent experiments
88 have shown that when *Pou4f2* and *Isl1* are misexpressed under the control of the
89 endogenous *Atoh7* promoter, this is sufficient to fully rescue the defects in RGC
90 development seen in *Atoh7* mutants (Gan et al., 1999; Pan et al., 2008; Wu et al.,
91 2015). This implies that *Atoh7* may act permissively to enable expression of these two
92 factors in early-stage RPCs.

93 Other data, however, suggest that *Atoh7* may not be essential for RGC
94 specification. Previous studies indicate that immature RGCs are present in *Atoh7*-
95 deficient mice in embryonic retina, although at reduced numbers relative to controls
96 (Brown et al., 2001; Brzezinski et al., 2012). Genetic fate mapping studies further raise
97 questions about the necessity of *Atoh7* for RGC specification. Analysis of *Atoh7-Cre*
98 knock-in mice reveal that only 55% of all RGCs are generated from *Atoh7*-expressing
99 RPCs (Brzezinski et al., 2012; Feng et al., 2010; Poggi et al., 2005). Although this
100 outcome may reflect inefficient activation of Cre-dependent reporter constructs, it may
101 also imply that a subset of RGCs are specified through an *Atoh7*-independent
102 mechanism that requires trophic support from *Atoh7*-expressing RPCs or *Atoh7*-derived
103 RGCs.

104 To distinguish the role of *Atoh7* in controlling RGC specification and survival, we
105 prevented RGC death in *Atoh7*-deficient mice by simultaneously inactivating the
106 proapoptotic gene *Bax* (Chen et al., 2013; Knudson et al., 1995). Strikingly, we
107 observed only a 25.2±0.9% reduction in adult RGC numbers in *Atoh7*^{-/-};*Bax*^{-/-} retinas
108 relative to *Bax*^{-/-} controls. While mutant RGCs showed severe defects in formation of
109 axonal projections and retinal vasculature, we found that ‘rescued’ RGCs expressed
110 both *Pou4f2* and *Isl1* in addition to other markers of terminal differentiation, fired action

111 potentials in response to light, and formed functional synapses with retinal neurons.
112 Single-cell RNA-Sequencing (scRNA-Seq) analysis of *Atoh7*;*Bax*-deficient retinas
113 shows that although RGC differentiation is delayed relative to wildtype, *Atoh7*-deficient
114 RGCs express both *Pou4f2* and *Isl1*. This study demonstrates that, while *Atoh7* is
115 required for both terminal differentiation and survival of RGCs, it is not necessary for
116 specification of the majority of RGCs.

117

118

119 **Experimental Procedures**

120

121 **Mice**

122 Animals were housed and treated in accordance with NIH and IACUC guidelines, and
123 used protocols approved by the Johns Hopkins University Animal Care and Use
124 Committee (Protocol numbers MO16A212). *Atoh7*^{Cre/Cre} mice are a knock-in line where
125 Cre recombinase replaced the entire *Atoh7* gene and was a gift from Dr. Lin Gan
126 (referred to as *Atoh7*^{-/-}) (Yang et al., 2003) (RRID:MGI:3717726). The *Bax*^{tm1Sjk/tm1Sjk}
127 (*Bax*^{-/-}) mice containing a neomycin cassette that replaces critical exons 2-5, were
128 purchased from The Jackson Laboratory (Knudson et al., 1995)(JAX:002994,
129 RRID:IMSR_JAX:002994). The *Bax*^{-/-} mice are unpigmented since the *Bax* gene is
130 linked to the *Tyrosinase* (*Tyr*) and *Pink-eyed dilution* (*p*) gene by 21cM and 5cM
131 respectively. The conditional *Bax*^{tm2Sjk/tm2Sjk} (*Bax*^{fl/fl}) mice containing a LoxP sites
132 flanking exons 2-4, were purchased from The Jackson Laboratory (JAX:006329,
133 RRID:IMSR_JAX:006329) (Takeuchi et al., 2005). *Atoh7*^{tTA/tTA};B&I-EE mice are a
134 combination of two genetic strains. In the first strain (*Atoh7*^{tTA/tTA}), the tetracycline-
135 responsive artificial transcription factor tTA replaces the *Atoh7* gene. In the absence of
136 tetracycline, the tTA activates the tetracycline responsive element which is driving the
137 expression of *Brn3b* and *Isl1* in the second strain (B&I-EE). Therefore, in effect the
138 *Atoh7* promoter will drive the expression of *Brn3b* and *Isl1*. This mouse line has been
139 previously reported to rescue all reported effects of *Atoh7* loss of function, and was a
140 gift from Dr. Xiuqian Mu (Wu et al., 2015)(MGI:5749708 and MGI:5749713). The
141 *Crx*>*Atoh7* mice, a transgene that expresses the full-length *Atoh7* coding sequence

142 under the control of the *Crx* promoter, was previously published (Prasov and Glaser,
143 2012) (MGI:5433215). A tdTomato Cre recombinase reporter mouse *Rosa26^{tdTomAi14}*
144 (JAX:007914, RRID:IMSR_JAX:007914) (Madisen et al., 2010) was used to label cells
145 in a Cre recombinase-dependent manner. The *Chx10-Cre* mouse line is a transgenic
146 line purchased from The Jackson Laboratory, originally developed by Constance
147 Cepko's laboratory (JAX:005105, RRID:IMSR_JAX:005105) (Rowan and Cepko, 2004),
148 expresses Cre recombinase broadly in all retinal progenitor cells from E10-E15.5. The
149 *Opn4^{taulacZ}* mice were used to trace the ipRGC projections to the brain (Hattar et al.,
150 2002). Throughout the manuscript, controls are heterozygous for both *Atoh7* and *Bax*
151 (*Atoh7^{+/-};Bax^{+/-}*), whereas *Atoh7^{-/-}* mice were also are heterozygous for *Bax* (*Atoh7^{-/-};*
152 *Bax^{+/-}*).

153

154 **Statistics**

155 All statistical tests, apart from analysis of the scRNA-seq data, were performed in
156 Graphpad Prism 6 (RRID:SCR_002798). The statistical tests used are listed in figure
157 captions.

158

159 **Immunohistochemistry**

160 Adult retinas from P40-P200 mice were obtained from by enucleating whole eyes, fixing
161 for 30 minutes in 4% paraformaldehyde (PFA) diluted in PBS, dissecting to remove the
162 cornea and lens, dissecting the retina from the RPE, and antibody staining proceeded in
163 a 24-Multiwell Cell Culture Plate (Corning #353047). Retinas were blocked in 500 μ l of
164 PBS containing 0.3% Triton X100 and 6% goat serum for 2 hours at room temperature.
165 Several antibodies were used in this study (dilutions are between brackets): Mouse
166 IgG1 anti-Brn3a (Millipore Cat# MAB1585 RRID:AB_94166) (1:250), Rabbit anti-
167 RBPMS (GeneTex Cat# GTX118619 RRID:AB_10720427) (1:250), Rabbit anti Brn3b
168 (Badea et al., 2009) (1:100), Mouse anti-Smi32 (non-phosphorylated anti-Neurofilament
169 H (NF-H)) (BioLegend Cat# 801701 RRID:AB_2564642) (1:500), Mouse anti
170 Neurofilament Medium (Thermo Fisher Scientific Cat# 13-0700, RRID:AB_2532998)
171 (1:500), Chicken anti Neurofilament Heavy (Millipore Cat# AB5539,
172 RRID:AB_11212161) (1:250), Mouse anti-GFAP (Sigma-Aldrich Cat# C9205

173 RRID:AB_476889) (1:1000), GS-IB4 (Molecular Probes Cat# I21411 also I21411
174 RRID:AB_2314662) (1:250), Rabbit anti-Pax2 (BioLegend Cat# 901001
175 RRID:AB_2565001) (1:100), Mouse anti-Tuj1 (R and D Systems Cat# MAB1195
176 RRID:AB_357520) (1:200), Rabbit anti DsRed Takara Bio (Cat# 632496,
177 RRID:AB_10013483) (1:250), Mouse anti-Islet1 (DSHB Cat# 40.2D6 RRID:AB_528315)
178 (1:200), and Rabbit anti-Opn4 (Advanced Targeting Systems Cat# UF006,
179 RRID:AB_2314781) (1:500). The appropriate antibodies were diluted in blocking
180 solution and incubated for two days at 4°C. Retinas were then washed in three changes
181 of PBS, 15 minutes each, then placed in the appropriate Alexa Fluor secondary
182 antibody (Invitrogen) (1:500) overnight at 4°C. Retinas were washed in 200 µl of PBS
183 containing 1xDAPI, then washed three times in PBS for 15 minutes each, and mounted
184 flat on slides in VectaShield (Vector Labs, RRID:AB_2336789). Regionalized dissections
185 were done as follows; before enucleation, the most nasal part of the sclera was marked
186 with a cauterizer. This mark was used during the dissection to make a marking incision
187 into the retina, following the above staining protocol. Retinas were imaged on a Zeiss
188 LSM 700 or 800 Confocal at the Johns Hopkins University Integrated Imaging Center
189 Core Facility (RRID:SCR_016187).

190 For embryonic studies, developing embryos harvested at E12.5 and E14.5 were
191 washed in a Petri dish with sterile PBS three times for 10 minutes. Tail was used for
192 genotyping. The heads were fixed in 4% PFA for 30 minutes and then cryoprotected in
193 30% sucrose at 4°C overnight, frozen in OCT, and sectioned at 18 µm thickness using a
194 cryostat. Sections were dried at 30°C for 15 minutes and then washed 10 minutes in
195 three changes of PBS. Sections were then blocked and stained as above in a
196 humidified chamber overnight. Sections were then mounted and imaged as described.

197

198 **Cell density analysis**

199 All cell counting was done manually. To confirm the reproducibility of the cell counts,
200 randomly selected selections from each sample were counted twice, and counts were
201 consistently found to be essentially identical. Density was calculated as the number of
202 cells per area. All measurements and cell number analysis was done manually in
203 ImageJ (Fiji, RRID:SCR_002285) and Adobe Photoshop CS6 (RRID:SCR_014199).

204 In adult flat-mounted retinas, density of RGCs was calculated by obtaining at
205 least 4 representative images at 40x of 600 μm x 600 μm with 1 μm optical sections.
206 Optical sections were projected together with maximum intensity, including cells only in
207 the retinal layers of interest. Representative images were taken similarly across all
208 genotypes without *a priori* knowledge of the genotype. However, some genotypes
209 contain marked phenotypic differences, which include a pigment mutation linked to the
210 *Bax* locus, drastic RGC number reduction as in the *Atoh7^{-/-}*, and/or misguided axons.

211 Three representative areas of each retina were averaged for the density analysis

212 In E12.5 embryonic retinal sections, a representative confocal image was taken
213 at 40x of 600 μm x 600 μm with optical sections of 1 μm projected together with
214 maximum intensity. The sections chosen for analysis were all positive for Pax2+ optic
215 nerve head cells, as the central retina contains the earliest-born RGCs. At least two
216 sections with matching criteria were analyzed for each E12.5 embryo. Density was
217 calculated by dividing the number of Brn3a+ RGCs and dividing by the area of the
218 retina. To limit the analysis to the RGC neurogenic zone, we limited the quantification to
219 the leading edge of RGC genesis. The percentage of mature Brn3a+ RGCs at E12.5
220 were determined by counting their number within the ganglion cell layer (GCL). The
221 number of mature Brn3a+ RGCs was then divided by the number of total Brn3a+ RGCs
222 in a section, and then averaged across all sections. This ratio represents the number of
223 Brn3a+ RGCs already in the nascent ganglion cell layer versus RGCs migrating through
224 the neuroblast layer to the ganglion cell layer.

225

226 **Multielectrode array recordings**

227 Mice were dark-adapted for 1-2 hours before being sacrificed and dissected under dim
228 red light. Retinas were isolated in Ames' medium (Sigma) bubbled with 95% O₂/5% CO₂
229 (carbogen) at room temperature, trimmed into small rectangles, and then placed on a
230 6x10 perforated multielectrode array (Multichannel Systems, Tübingen, Germany),
231 ganglion-cell-side down. Tissue was perfused with Ames' bubbled with carbogen and
232 kept at 32°C throughout the experiment. Data acquisition was performed using the
233 MC_Rack software (ALA Scientific Instruments, Inc.), at a 50-kHz sampling rate. An
234 offline spike sorter (Plexon Inc) was used for spike sorting.

235 UV stimuli ($I_{\text{mean}} \approx 5 \times 10^3$ photons/cm²/s, 398 nm) were generated through a
236 modified DLP projector (HP Notebook Projection Companion Projector, Model: HSTNN-
237 FP01) (frame rate = 60 Hz) and were delivered through an inverted microscope
238 objective. All stimuli were programmed using the *Psychophysics Toolbox* in Matlab (The
239 Mathworks, Natick, MA). Stimuli include: (1) 120-s, 1-Hz full-field square-wave flash
240 (100% Michelson contrast); (2) 10-min Gaussian white noise (GWN) flickering
241 checkerboard (pixel size = 44.77 μm); (3) 10-min spatially correlated “cloud” stimulus
242 that was generated by low-pass filtering the GWN. The cloud stimulus introduced dark
243 and bright areas of a range of scales within each frame, with the purpose of driving
244 large spatial receptive fields.

245 Analysis was first performed using custom-written Matlab (MATLAB R2014b)
246 codes, the results later were exported and edited in Adobe Illustrator CS6. For each
247 cell, the peristimulus time histogram (PSTH) of responses to square-wave flash was
248 calculated using 10-ms bins. Spatial and temporal receptive fields were identified based
249 on noise data using a nonlinear model previously described in detail (McFarland et al.,
250 2013; Shi et al., 2019).

251 Fewer cells were recorded from *Atoh7^{Cre/Cre};Bax^{-/-}* mice compared to the wildtype
252 and *Bax^{-/-}*, as the nerve fiber layer (NFL) and retinal vasculature are improperly
253 developed and thus provide an insulating layer that needs to be removed in order to
254 obtain high quality recordings. No cells were recorded from *Atoh7^{Cre/Cre}* mice, due to the
255 >99% reduction in RGC numbers.

256

257 **Pupillary light response (PLR)**

258 PLR experiments were performed on mice that were dark adapted for at least 1 hour
259 prior to any experiment. PLR was measured by gently restraining the mice by hand
260 (without anesthesia) and exposing them to a cool white light LED bulb (6500K, light
261 intensity: 15 W/m², MR16, SuperBrightLEDs.com) that was directed at one eye using a
262 gooseneck arm of a dissecting microscope light source. Constriction of the pupil was
263 recorded using a Sony Handycam camcorder (FDRAX33) from either the contralateral
264 or ipsilateral eye to the light source. The baseline pupil size of each mouse was first
265 recorded for at least 5 seconds using an infrared light source, following which the white

266 LED bulb was turned on for at least 30 seconds. Video recordings were analyzed by
267 creating screen shot images in Joint Photographic Experts Group format (jpg) of the
268 pupil prior to and during light stimulation using VLC media player
269 (<https://www.videolan.org/vlc/>). Pupil area was then quantified in ImageJ (Fiji,
270 RRID:SCR_002285). To determine the relative pupil area, pupil size during the light
271 stimulation was divided by pupil size prior to light stimulation.

272

273 **Tissue dissociation for generation of single-cell suspensions**

274 Eyes were enucleated from E14 time-pregnant animals and placed directly into ice-cold
275 1X PBS. Retinas were dissected in cold 1X PBS, and then placed into 200 μ l of cold
276 HBSS per 2-3 retinas. Tissue dissociation was induced through addition of an
277 equivalent volume of Papain solution (1 ml - 700 μ l reagent grade water, 100 μ l fresh 50
278 mM L-Cysteine (Sigma), 100 μ l 10 mM EDTA, 10 μ l 60 mM 2-mercaptoethanol (Sigma),
279 with Papain added to 1 mg/ml (1:10 dilution of 10 mg/ml Papain solution; Worthington)).
280 Papain-retina mixture was placed at 37°C for 10 minutes with slight trituration every 2-3
281 minutes. Enzymatic dissociation was halted through addition of 600 μ l of Neurobasal
282 Media + 10% FBS for every 400ul of dissociation solution. DNA from lysed cells was
283 removed using 5 μ l RNase-free DNase I (Roche) for every 1 ml dissociate and
284 incubated at 37°C for 5 minutes, followed by slight trituration using a 1 ml pipette. Cells
285 were pelleted after centrifugation (300 RCF for 5 minutes at 4°C), followed by
286 resuspension in 2-3 ml of Neurobasal media supplemented with 1% FBS. The final
287 solution was passed through a 50 μ m filter to remove cellular aggregates and
288 undissociated debris.

289

290 **10x Genomics Sequencing and Analysis**

291 Single cell RNA sequencing of dissociated retinal cells from E14 *Atoh7*^{-/+};*Bax*^{-/-} and
292 *Atoh7*^{-/-};*Bax*^{-/-} was performed using 10x Genomics Chromium 3' v2 platform (PN-
293 120223) (Pleasanton, CA), followed by sequencing using the NextSeq500 platform with
294 default 10x sequencing parameters (R1 - 26bp; R2 - 98bp; i7 - 8bp). Single-cell analysis
295 of the wildtype (WT) E14 developing mouse retina was obtained from previously

296 reported samples obtained from GEO (GSE118614); data obtained using similar
297 isolation protocols are described previously (Clark et al., 2019).

298

299 **Gene Set Usage Pattern Discovery with scCoGAPS**

300 CoGAPS v.3.5.6 (Sherman et al., 2019; Stein-O'Brien et al., 2019) was used to find
301 patterns of gene set usage by Neurogenic and Retinal Ganglion cells. The expression
302 matrix used as input was normalized to 10,000 counts per cell, subsetted down to 5235
303 most highly variable genes and log2 transformed. The CoGAPS parameters used are:
304 singleCell=TRUE, nPatterns = 30, nIterations = 50000, distributed = single-cell,
305 sparseOptimization = True, seed = 803L, and nSets = 10. The final number of patterns
306 stabilized at 31.

307

308 **Identification of *Atoh7*-Dependent Genes**

309 Data was subsetted down to neurogenic RPCs and RGCs. Monocle's differential gene
310 test was conducted between control (WT and *Bax*^{-/-}) and *Atoh7* mutants (*Atoh7*^{-/-} *Atoh7*^{-/-}
311 *;**Bax*^{-/-}) as such:

```
312 differentialGeneTest(dat[genes expressed in >=10 cells], fullModelFormulaStr =  
313 '~(Atoh7 genotype) + Total_mRNAs', reducedModelFormulaStr =  
314 '~Total_mRNAs',cores=4).
```

315

316 **Pseudotime Analysis between Genotypes**

317 Scanpy v1.4 (Wolf et al., 2018) was first used to assign diffusion pseudotime values
318 (Haghverdi et al., 2016) to cells in the retinal ganglion cell trajectory. Cell types included
319 in this final dataset were restricted to retinal ganglion cells, primary RPCs and
320 neurogenic RPCs. To preprocess the dataset, genes <10 counts were removed, and the
321 expression matrix was normalized to 10,000 counts/cell and log-transformed. Highly
322 variable genes used for ordering were identified using Scanpy's `highly_variable_genes``
323 function with default parameters except `flavor='cell_ranger'` and `n_top_genes=3000`. 50
324 principal components were calculated using default PCA parameters with
325 `random_state=123456`. To compute the neighborhood graph with the batch effect of
326 genotype removed, we used BBKNN with `batch_key = "Genotype"` and

327 neighbors_within batch=3 (Polański et al., 2019). 10 diffusion components were then
328 computed and used for input to assign diffusion pseudotime values with an RPC cell as
329 root. To find genes differentially expressed between the developmental trajectories of
330 the WT and *Atoh7^{Cre/Cre};Bax^{-/-}* genotypes, Monocle's differential gene test (Qiu et al.,
331 2017; Trapnell et al., 2014) was performed in R, on neurogenic RPCs and RGCs of WT
332 and *Atoh7^{Cre/Cre};Bax^{-/-}* null genotypes:

```
333     differentialGeneTest(dat[genes expressed in >=10 cells], fullModelFormulaStr =  
334     '~sm.ns(Pseudotime,df=3)*Genotype+Total_mRNAs', reducedModelFormulaStr  
335     = '~sm.ns(Pseudotime,df=3)+Genotype+Total_mRNAs',cores=3)
```

336

337 **Data Availability**

338 Processed (expression, gene (featureData), and cell (phenoData) matrices) and raw
339 sequence information (.bam files) are available for direct download through GEO
340 GSE148814.

341

342 ***In situ* Hybridization**

343 Developing embryos harvested at E14.5 were washed in petri dishes filled with sterile
344 DEPC-treated PBS at least three times. The head of the embryo was plunged into OCT
345 and then immediately frozen and stored at -80°C until needed, and the tail was used for
346 genotyping. 20 µm sections were taken using a cryostat. Sections were allowed to dry
347 to slides for a few hours and then were stored at -80°C until needed. *In situ* hybridization
348 was performed as previously described (Shimogori et al., 2010).

349

350

351 **Results**

352

353 ***Atoh7* promotes RGC survival, but RGC specification is largely *Atoh7*- 354 independent**

355 In the absence of *Atoh7*, there is an increase in apoptosis of both *Atoh7*-derived cells
356 across embryonic retinal development and non-*Atoh7*-derived cells in the GCL at E16.5
357 and E17.5 (Feng et al., 2010; Prasov and Glaser, 2012). These data suggest that *Atoh7*

358 may promote RGC survival in both a cell-autonomous and cell non-autonomous
359 manner. To better understand the role *Atoh7* plays in RGC development, independent
360 of its role in RGC survival, we disrupted both *Atoh7* and the proapoptotic *Bax* gene, in
361 order to inhibit apoptosis in the retina.

362 We used *Atoh7^{Cre/Cre}* mice, in which the *Atoh7* coding sequence is replaced with
363 Cre recombinase via targeted recombination, generating a null allele, to analyze *Atoh7*
364 function (Yang et al., 2003). We first examined the expression of RBPMS and *Isl1*, both
365 of which are broadly expressed in RGCs, in *Atoh7^{Cre/Cre};Bax^{-/-}* mice (Figure 1A-C)
366 (hereafter referred to as *Atoh7^{-/-};Bax^{-/-}* mice). *Isl1*, a LIM family homeodomain
367 transcription factor, is necessary for RGC development and maintenance in adulthood
368 (Mu et al., 2008; Pan et al., 2008), and is expressed in mature RGCs, bipolar, and
369 amacrine cells. Using anti-Rbpms to selectively label all RGCs, we observed an
370 $159\pm 7\%$ (Figure 1A,B) increase in RGC number in *Bax^{-/-}* retinas, in line with previous
371 results indicating that RGCs undergo extensive levels of apoptosis during development.
372 However, we observe only a $25.2\pm 0.9\%$ reduction in RGCs in *Atoh7^{-/-};Bax^{-/-}* relative to
373 *Bax^{-/-}* retinas. This contrasts with the $99.54\pm 0.12\%$ reduction in RGCs in the *Atoh7^{-/-}*
374 compared to controls (Figure 1A,B). Similar results were observed for anti-*Isl1* cells in
375 the GCL (Figure 1A,C).

376 Specific RGC markers, *Brn3a* (*Pou4f1*) and *Brn3b* (*Pou4f2*), were used to
377 quantify RGCs. For wildtype and *Bax^{-/-}* lines, *Brn3a* and *Brn3b* numbers were similar to
378 published reports (Figure 1D-F) (Prasov and Glaser, 2012; Rodriguez et al., 2014;
379 Wang et al., 2001; Xiang et al., 1995). In the *Atoh7^{-/-}* line a $99.72\pm 0.17\%$ reduction in
380 *Brn3a* RGC density was observed. However, in *Atoh7^{-/-};Bax^{-/-}* mice, the *Brn3a* RGCs
381 were substantially rescued in the *Atoh7^{-/-}* background and remained into adulthood.
382 $74.7\pm 0.9\%$ of *Brn3a* RGCs are rescued in *Atoh7^{-/-};Bax^{-/-}* mice relative to *Bax^{-/-}* levels in
383 adult, and RGCs display normal distribution across the entire retina (Figure 1D,E,
384 Supplemental Figure 1A,A'). Interestingly, *Brn3b* RGCs were also increased in *Atoh7^{-/-}*
385 *;Bax^{-/-}* relative to *Bax^{-/-}* retinas, but to a lesser extent than the *Brn3a* ($28.8\pm 5.9\%$; Figure
386 1D,F, Supplemental Figure 1B,B'). Expression of *Isl1*, *Brn3b*, and, to a lesser extent
387 *Brn3a* have previously reported to require *Atoh7* (Mu et al., 2008, 2004, 2005; Pan et
388 al., 2008; Rodriguez et al., 2014; Wu et al., 2015). However, our data indicates that the

389 expression of *Isl1*, *Brn3a*, and *Brn3b* expression in RGCs can occur independent of
390 *Atoh7* (Figure 1).

391 To investigate the extent to which rescued RGCs resembled wildtype neurons,
392 we examined the expression of markers of major classes of mature RGCs. We
393 investigated the prevalence of intrinsically photosensitive RGCs (ipRGCs) within *Atoh7*^{-/-}
394 *;Bax*^{-/-} retinas. During the development of ipRGCs, a majority of cells express *Brn3b*,
395 although in some cases only transiently (Chen and Hattar, 2012). To determine the
396 percentage of rescued ipRGCs in *Atoh7*^{-/-}*;Bax*^{-/-} mice, we used a melanopsin antibody
397 that predominantly labels the high melanopsin-expressing M1 and M2 ipRGC
398 populations. We observe that 34.1% of ipRGCs are rescued in *Atoh7*^{-/-}*;Bax*^{-/-} mice
399 relative to *Bax*^{-/-}, proportions similar to the fraction of *Brn3b*-positive RGCs in wildtype
400 (Figure 1D,F, Supplemental Figure 1C-F). This indicates that *Brn3b*-positive ipRGCs
401 differentiate normally in the absence of *Atoh7*.

402 To eliminate the possibility that global loss of function of *Bax* caused a non-
403 specific rescue of RGC development, we tested the effects of retina-specific conditional
404 mutants of *Bax*. Using *Chx10-Cre;Atoh7*^{Cre/Cre}*;Bax*^{fl/fl} (Figure 1G,H). In this model, *Bax* is
405 selectively disrupted in RPCs beginning at E10-10.5 (Rowan and Cepko, 2004).
406 Removal of *Bax* from all RPCs shows RGC development to the same extent as in
407 *Atoh7*^{Cre/Cre}*;Bax*^{-/-} (Figure 1G, H). In contrast, *Atoh7*^{Cre/Cre}*;Bax*^{fl/fl} mice did not show any
408 detectable rescue of RGC development (Figure 1G,H). This suggests that the survival-
409 promoting effects of *Atoh7* may take place only during a narrow temporal window, and
410 that Cre-mediated deletion of *Bax* in the *Atoh7*^{Cre/Cre} line may not occur rapidly or
411 efficiently enough to rescue RGC development.

412 In both wildtype and *Atoh7*^{-/-}*;Bax*^{-/-} animals, 34±11% and 34±26% of RGCs are
413 derived from *Atoh7*-expressing cells, a finding which independently confirms similar
414 lineage tracings in previous studies (Supplemental Figure 2A-C) (Brzezinski et al.,
415 2012; Feng et al., 2010). This indicates that while RGCs that are normally derived from
416 *Atoh7*-expressing neurogenic RPCs are reduced in the absence of *Atoh7*, *Atoh7* is not
417 required for their specification. Furthermore, RGCs that are derived from non-*Atoh7*-
418 expressing RPCs require *Atoh7* for survival through a non-cell autonomous mechanism.

419

420 **RGCs specified in the absence of *Atoh7* generate light-driven photic responses**
421 **transduced from the outer retina**

422 As we determined that loss of Bax in the *Atoh7*^{-/-} background rescues RGC
423 specification, we next sought to investigate the degree to which RGCs differentiated in
424 the absence of *Atoh7*. One hallmark of RGC differentiation is formation of presynaptic
425 contacts and the generation of light-induced action potentials. We used multi-electrode
426 array recordings to test the light responsiveness of the RGCs within the various mutant
427 models. Spatiotemporal noise stimuli were used to activate the retina with a mean
428 excitation of 398 nm ($I_{\text{mean}} \approx 5 \times 10^3$ photons/cm²/s), a wavelength that predominantly
429 activates S-cones (Wang et al., 2011), and does not activate Opn4-expressing ipRGCs
430 (Berson et al., 2002; Do, 2019; Do and Yau, 2010; Hattar et al., 2002). We observed an
431 identical stimulus-response profile in wildtype and *Bax*^{-/-} RGCs (Figure 2A): an expected
432 result given that *Bax*^{-/-} mice display normal visual responses within the Morris water
433 maze test (Chen et al., 2013). The population of RGCs included cells with similar light-
434 evoked responses as ON sustained, ON transient, ON/OFF transient, OFF transient, or
435 OFF sustained RGCs (Figure 2A, Supplemental Figure 3). The spatial receptive fields of
436 the *Atoh7*^{-/-};*Bax*^{-/-} RGCs were also slightly smaller than normal (*Atoh7*^{-/-};*Bax*^{-/-}
437 average 191±37.1 μm [n=56 from 8 mice], control average 212±42.3 μm [n=92 from 5
438 mice] and *Bax*^{-/-} average 223±42.4 μm [n=96 from 4 mice]) (Figure 2B) and the kinetics
439 of the light-driven responses in the *Atoh7*^{-/-};*Bax*^{-/-} retinas slightly slower than those of
440 control and *Bax*^{-/-} cells (Figure 2C).

441 The linear analysis used for the MEA data can only reveal an averaged STA,
442 which could be a compression of multiple receptive fields. Thus those having multiple
443 receptive fields such as ON-OFF cells could potentially be hidden with this method and
444 require more sophisticated analysis strategy (Shi et al., 2019). However, diversity in
445 PSTH profiles is clearly observed, suggesting that different cell types coexist in *Atoh7*^{-/-}
446 *Bax*^{-/-} mice. We chose not to perform more complex analysis such as classifying each
447 cell into known cell types due to the relatively small sample size. These will be intriguing
448 questions to address when larger amounts of data are made accessible. The near-
449 normal properties of the *Atoh7*^{-/-};*Bax*^{-/-} RGCs show that RGCs present in the *Atoh7*^{-/-}

450 $^{-/-};Bax^{-/-}$ are wired properly to the outer retina, specifically the S-cones, and receive
451 normal circuit input.

452

453 **RGC axon guidance is *Atoh7*-dependent**

454 While RGCs in *Atoh7* $^{-/-};Bax^{-/-}$ animals appropriately respond to detection of visual stimuli
455 by the outer retina, the ability of these RGCs to form postsynaptic connections in the
456 brain is compromised. We observed a substantially reduced pupillary light response in
457 *Atoh7* $^{-/-};Bax^{-/-}$ animals compared to controls (Figure 3B,C). To determine the cause of
458 behavioral deficits, we assessed the distribution of RGC axons. Immunostaining for
459 Smi32 (non-phosphorylated Nfh) (Figure 3A,D, Supplemental Figure 4C), Nfh, and Nfm
460 (Supplemental Figure 4A,B) was used to evaluate RGC axonal integrity, and showed
461 normal architecture in wildtype and *Bax* $^{-/-}$ mice. We were surprised to find that the <1%
462 of RGCs that survive in the *Atoh7* $^{-/-}$ showed severe guidance defects, in that they
463 fasciculate, come in close proximity to where the optic disc should be, seem to
464 overshoot the optic disc, and then continue to extend within the retina. The great
465 majority of *Atoh7* $^{-/-}$ RGC axons fail to correctly target the optic disc, with only a few
466 axons exiting and forming a rudimentary optic nerve, leading to a severely reduced PLR
467 (Figure 3B,C, Supplemental Figure 5A-C). The gross misguidance of axons was also
468 observed in *Atoh7* $^{-/-};Bax^{-/-}$ mice (Figure 3A,D). As in *Atoh7* $^{-/-}$ mice, RGC axons in *Atoh7* $^{-/-};Bax^{-/-}$
469 mice fasciculate, fail to correctly target the optic disk, and form tracts that extend
470 around the retina, with only rudimentary optic nerves observed. This guidance defect
471 did not result from defective formation of the optic disc, as Pax2-positive glial cells that
472 mark this structure are present in the *Atoh7* $^{-/-};Bax^{-/-}$ at E12.5 (Supplemental Figure 6A,B)
473 and E14.5 (data not shown). This demonstrates that the optic disk is present, but the
474 axons are unable to find their way out of the retina in large numbers. These findings are
475 reminiscent of previous reports in zebrafish, in which morpholino-mediated disruption of
476 *atoh7* expression in early-stage RPCs disrupted the correct targeting of axons of later-
477 born, *atoh7*-positive RGCs to the optic tectum (Pittman et al., 2008).

478 Previous studies have observed a lack or massive reduction in physical or
479 functional connection to the brain in *Atoh7* $^{-/-}$ mice (Brown et al., 2001; Brzezinski et al.,
480 2005; Triplett et al., 2011; Wee et al., 2002). Consistent with the failure of mutant RGCs

481 to correctly target the optic nerve, we observe severe disruptions in behavioral
482 responses to light in *Atoh7^{-/-};Bax^{-/-}* mice that are essentially indistinguishable from those
483 seen in *Atoh7^{-/-}* mice. *Atoh7^{-/-};Bax^{-/-}* mice show no detectable optokinetic response
484 (Supplemental Figure 7A), and show no visual cue-dependent reduction in escape time
485 during successive trials of the Morris water maze (Supplemental Figure 7B). *Opn4:Tau-*
486 *lacZ* knock-in mice, which visualize the axonal projections of M1 ipRGCs, show no
487 detectable signal in the brain (Supplemental Figure 7C) (Hattar et al., 2002). Intraocular
488 injection of fluorescently-labeled cholera toxin beta, which visualizes RGC axonal
489 terminals (Bedont et al., 2014), likewise shows no brain labeling in both *Atoh7^{-/-};Bax^{-/-}*
490 and *Atoh7^{-/-}* mice (Supplemental Figure 7D). However, while the contralateral PLR is
491 significantly reduced compared to wildtype in both the *Atoh7^{-/-}* and *Atoh7^{-/-};Bax^{-/-}* mice, it
492 is nonetheless detectable, indicating that a small number of RGC axons target the
493 olivary pretectal nucleus in *Atoh7^{-/-};Bax^{-/-}* mice, although we are unable to detect these
494 using standard techniques (Figure 3B,C, Supplemental Figure 5 A-C).

495

496 **Retinal vasculature development is disrupted in the absence of *Atoh7***

497 In both mice and humans, loss of *Atoh7* expression results in persistence of the hyaloid
498 vasculature (Edwards et al., 2012; Ghiasvand et al., 2011; Kondo et al., 2016; Prasov et
499 al., 2012). The persistence of the hyaloid vasculature in *Atoh7^{-/-}* retinas until P14 was
500 previously observed. We likewise observe persistence of the hyaloid vasculature into
501 adulthood in *Atoh7^{-/-}* retinas (Figure 3E), which is observed even in one year old mice
502 (data not shown). Surprisingly, even with the rescue of a majority of Brn3a RGCs in
503 *Atoh7^{-/-};Bax^{-/-}* animals, the hyaloid vasculature still fails to regress (Figure 3E). Likewise,
504 *Crx>Atoh7;Atoh7^{-/-}* mice, in which *Atoh7* is misexpressed in photoreceptor precursors,
505 also fail to induce hyaloid regression (Figure 3E). This is in sharp contrast to the
506 rescued vascular phenotype observed in *Atoh7^{tTA/tTA};B&I-EE* mice, when *Brn3b* & *Isl1*
507 are ectopically expressed from the endogenous *Atoh7* locus in a *Atoh7*-deficient mouse
508 using the tet-off system (Wu et al., 2015)(Figure 3E), and implies that *Brn3b* and *Isl1*
509 may activate expression of secreted factors that drive vascular regression.

510

511

512 **RGC differentiation is delayed in the absence of *Atoh7***

513 In order to examine potential differences in RGC development within *Atoh7*^{-/-} and *Atoh7*^{-/-};
514 *Bax*^{-/-} compared to wildtype and *Bax*^{-/-} control animals, we next performed single-cell
515 RNA-sequencing on *Bax*^{-/-}, *Atoh7*^{-/-}, and *Atoh7*^{-/-};*Bax*^{-/-} retinas to more comprehensively
516 profile changes in cell type specification and global transcriptional differences across
517 the genotypes. We profiled 67,050 E14.5 retinal cells from *Bax*^{-/-}, *Atoh7*^{-/-}, and *Atoh7*^{-/-};
518 *Bax*^{-/-} mice, and aggregated the datasets with 26,078 age-matched wildtype retinal
519 cells from previous studies (Clark et al., 2019) (Figure 4A,B, Supplemental Figure 8).
520 Consistent with the previous single-cell studies of E14 mouse retinal development
521 (Clark et al., 2019; Giudice et al., 2019), we observed a continuous manifold of cells,
522 from primary RPCs to neurogenic RPCs, leading to three separate differentiation
523 trajectories that give rise to RGCs, cones, and amacrine/horizontal cells, respectively
524 (Figure 4A-B; Supplemental Figure 9A). Analysis of the cell type proportions across the
525 genotypes revealed a depletion of RGCs in both *Atoh7* mutant samples (*Atoh7*^{-/-} and
526 *Atoh7*^{-/-};*Bax*^{-/-}) compared to both wildtype and *Bax*^{-/-} controls (Figure 4C-D,
527 Supplemental Figure 8E-F). This reduction in the number of RGCs included a
528 compensatory increase in cone photoreceptors and neurogenic RPCs, consistent
529 previous findings (Figure 4C-D, Supplemental Figure 8E-F) (Brown et al., 2001;
530 Brzezinski et al., 2005; Hufnagel et al., 2010; Wang et al., 2001).

531 Using the scRNA-seq data, we next assessed the differential gene expression
532 within neurogenic RPCs and RGCs across control -- WT and *Bax*^{-/-} -- and *Atoh7*-
533 deficient -- *Atoh7*^{-/-} and *Atoh7*^{-/-};*Bax*^{-/-} -- samples. Using strict differential expression
534 cutoffs (q-value < 1e-300), we identified 230 *Atoh7*-dependent differentially expressed
535 transcripts within neurogenic RPCs and RGCs (Supplemental Table 1). Genes enriched
536 within control samples (Figure 4E) highlighted many known factors in the specification
537 and differentiation of RGCs, including *Pou4f2* (*Brn3b*), *Isl1*, *Pou6f2* (*Rpf-1*), *Elavl4*,
538 *Gap43*, and *Irx2* (Choy et al., 2010; Ekström and Johansson, 2003; Kruger et al., 1998;
539 Zhou et al., 1996) (Supplemental Figure 9B). Conversely, differentially expressed
540 transcripts enriched in the *Atoh7* knockout samples (Figure 4E) were enriched for genes
541 involved in the Notch-signaling pathway -- *Rbpj*, *Dll3*, *Notch1*, and *Hes6* -- and for
542 transcripts enriched in neurogenic cells and photoreceptor precursors during retinal

543 development -- *Btg2*, *Neurog2*, *Bhlhe22*, *Insm1*, *Neurod1*, *Mybl1*, *Sstr2*,
544 *3110035E14Rik* (Clark et al., 2019; Supplemental Figure 9B).

545 *Atoh7*-deficient RGCs also show dramatically reduced expression of genes
546 known to regulate axon guidance including *Islr2*, which has been found to control RGC
547 axon fasciculation, as well as axon guidance at the optic chiasm (Panza et al., 2015). Of
548 particular interest are the observed increases in *Rbpj* and *3110035E14Rik* expressions
549 in *Atoh7*^{-/-} and *Atoh7*^{-/-};*Bax*^{-/-} samples. *Rbpj* is an upstream regulator of *Atoh7*
550 expression (Miesfeld et al., 2018b), and *3110035E14Rik* (*Vxn*), which functions similarly
551 to *Atoh7* by promoting retinal neurogenesis and early retinal cell fate specification
552 (Moore et al; 2018). The increased expression of transcripts that show enriched
553 expression in neurogenic cells and photoreceptor precursors is consistent with the
554 increase in generation of cone photoreceptor precursors seen in an *Atoh7*-deficient
555 retina. Combined with a recovery in the specification of RGC numbers in adult *Atoh7*
556 ^{-/-};*Bax*^{-/-} animals, these data suggest that loss of *Atoh7* expression leads to an increase
557 in expression of genes specific to neurogenic RPCs at the expense of RGC-enriched
558 transcripts, results consistent with a developmental delay.

559 In order to further assess the degree of an RGC-specific developmental delay we
560 performed pseudotemporal analyses using Scanpy (Wolf et al., 2018)(Figure 4F). We
561 observed a bias of the *Atoh7*-deficient cells within early pseudotime stages during RGC
562 differentiation. Both wildtype and *Bax*^{-/-} control cells had a broader distribution of cells
563 across pseudotime, results consistent with a failure of maturation or developmental
564 delay of RGC specification in *Atoh7*-deficient RGCs (Figure 4G). Differential expression
565 analysis assessing the changes in gene expression across the interaction of
566 pseudotime and genotypes revealed significant genotypic differences across RGC
567 development (Figure 4H; Supplemental Table 2).

568 In both *Atoh7*^{-/-} and *Atoh7*^{-/-};*Bax*^{-/-} samples, we observed a reduction of
569 expression in many genes enriched within mature RGCs -- *Pou4f2*, *Gap43*, *Sncg*, and
570 *Isl1*. We likewise observed reduced expression of a subset of genes in neurogenic
571 RPCs, including *Gal*. Increased expression of other genes predominantly expressed in
572 neurogenic RPCs -- including *Neurod1*, *Insm1*, *Neurod4*, *Hes6*, *Onecut1*, *Onecut2*, and
573 *Sox4* -- is observed in both *Atoh7*-deficient neurogenic RPCs and RGCs compared to

574 controls (Figure 4H). This implies that loss of function of *Atoh7* may delay differentiation
575 of RGCs from neurogenic RPCs. These results overall, closely match those obtained
576 from scRNA-Seq-based analysis of *Atoh7*^{-/-} retina conducted at E13.5 (Wu et al., 2020).

577 We next performed *in situ* hybridization to examine changes in global transcript
578 expression within the developing retina. RNA transcript expression was detected at
579 E14.5, at which point most RGCs are specified (Sidman, 1960; Young, 1985), and we
580 observed decreased expression of *Pou4f2* (*Brn3b*), *Isl1* and *Gal*, in both *Atoh7*^{-/-} and
581 *Atoh7*^{-/-};*Bax*^{-/-} mice, as determined by chromogenic *in situ* hybridization (Figure 4I).
582 Immunostaining of E14 retinas confirm a reduction in the number of cells
583 immunopositive for Brn3a (*Pou4f1*) and Brn3b (*Pou4f2*) (Figure 4J), as well as the pan-
584 RGC markers RBPMS and *Isl1* (Figure 4K), in the developing ganglion cell layer of
585 *Atoh7*-deficient retinas. At E12.5 we observed a marked decrease in both overall RGC
586 density and RGC number (Supplemental Figure 3). Together these results suggest that
587 loss of function of *Atoh7* delays RGC differentiation, and leads to an accumulation of
588 neurogenic RPCs.

589 To further identify patterns of temporal changes in gene expression across RGC
590 genesis between *Atoh7* mutant and control retinas, we performed the non-negative
591 matrix factorization technique scCoGAPS (Stein-O'Brien et al., 2019). Implementation of
592 scCoGAPS parses the gene expression into groups ('patterns') based on gene
593 expression profiles and without *a priori*, literature-based knowledge of gene interactions.
594 Using 5235 highly variable genes across the 29,182 neurogenic RPCs and
595 differentiating RGCs, we identified 31 patterns of gene expression (Supplemental Figure
596 10). These patterns correlated with both neurogenic RPCs – Patterns 6, 25, 5, 24, 11,
597 13, 18, 7, 21, 1, and 4 – and RGC – Patterns 8, 27, 28, 9, 26, 20, 22, 17, 31 – cell type
598 annotations and highlighted temporal changes in gene expression as assessed through
599 pseudotime analyses. Individual patterns - patterns 4, 5, and 29 - were highly correlated
600 with neurogenic cell annotations and *Atoh7*^{-/-} and *Atoh7*^{-/-};*Bax*^{-/-} genotypes, consistent
601 with a temporal delay in RGC specification, increased number of neurogenic cells, or
602 failure of cell type specification of neurogenic RPCs as a consequence of lost *Atoh7*
603 expression. Genes driving pattern 5 include genes enriched in early-stage neurogenic
604 RPCs, *Gadd45a* and *Sox11* (Clark et al., 2019).

605 Conversely, patterns highly correlated with RGC cell type annotations and
606 pseudotime – Patterns 9, 26, and 28 – had high correlation with control samples. The
607 most highly weighted genes in Patterns 9 and 26 are *Gap43* and *Igfbp1*, respectively,
608 which have been implicated in RGC axonal growth (Guo et al., 2018; Zhang et al.,
609 2000). Pattern 28 highlights cells towards the end of the RGC trajectory and is largely
610 driven by *Sncg*; a transcript enriched in most RGCs in the adult mouse retina (Soto et
611 al., 2008). The association of neurogenic patterns with *Atoh7*^{-/-} mutant retinas versus
612 those that highlight RGC differentiation and maturation patterns with control retinas
613 further support a developmental delay in mutant RGCs. Analysis of pattern marker
614 expression across the genotypes (Supplemental Figure 10) highlights both the temporal
615 delay and global changes in gene expression across the *Atoh7*^{-/-} mutant retinas
616 compared to controls.

617 Recent studies have comprehensively profiled RGC subtype diversity in the
618 mouse retina (Rheaume et al., 2018; Tran et al., 2019). However, these studies did not
619 characterize either the birthdates of individual RGC subtypes, or the transcriptional
620 networks controlling RGC subtype specification. Our combined data supports evidence
621 of both *Atoh7*-dependent and independent populations of RGCs. The delay in RGC
622 maturation and the failure of optic nerve formation seen in *Atoh7*-deficient retinas
623 suggest that the earliest pathfinding RGCs are *Atoh7*-dependent. We examined
624 expression of markers of mature RGC subtypes (Tran et al., 2019) within the developing
625 retina and correlated expression of the transcripts with RGC pseudotime (Supplemental
626 Figure 11) as many of the mature RGC subtype markers are not specific to RGCs. We
627 detected expression of selective markers for a fraction of mature RGC subtypes within
628 the E14.5 scRNA-seq dataset. Of transcripts in which readily detectable expression was
629 observed, many – including *Igfbp4*, *Foxp1*, *Stxbp6*, *Bhlhe22*, *Penk* – also display
630 enriched expression in primary or neurogenic RPCs. Expression of some markers of
631 RGC development and maturation – *Ebf3*, *Pou4f1*, *Pou4f2*, *Prdm8* and *Slc17a6* –
632 correlated well with pseudotemporal ordering and were depleted in *Atoh7*-deficient
633 RGCs. However, a limited number of RGC subtype markers, including *Irx3*, *Calb2*, and
634 *Tac1* were largely absent from *Atoh7* mutant RGCs.

635 Our data suggest the existence of *Atoh7*-dependent factors that promote both
636 RGC survival and pathfinding, and also drive hyaloid vascular regression. These are
637 likely derived from *Atoh7*-expressing neurogenic RPCs and/or RGCs. Factors mediating
638 hyaloid regression are likely to be secreted, while those regulating RGC survival could
639 also potentially act through contact-mediated signaling. Few annotated secreted
640 proteins show clear *Atoh7*-dependent expression in our scRNA-Seq dataset. The
641 neuropeptide galanin (*Gal*), which is strongly expressed in both neurogenic RPCs and
642 RGCs, was by far the most differentially expressed secreted factor in *Atoh7*-deficient
643 mice (Figure 4E,I, Supplemental Table 1). Galanin has been implicated in promoting the
644 survival of neural precursors (Cordero-Llana et al., 2014; Holmes et al., 2000), however,
645 *Gal*-deficient animals showed no differences in either the hyaloid vasculature regression
646 or RGC density, as compared to the control animals (Supplemental Figure 12).

647

648

649 Discussion

650 It is broadly accepted that *Atoh7* acts in RPCs as a competence factor that is essential
651 for RGC specification (Brzezinski et al., 2012; Mu et al., 2005; Yang et al., 2003; Baker
652 and Brown, 2018). In this study, though, we show that specification of the great majority
653 of RGCs occurs normally even in the absence of *Atoh7*. While RGC specification can
654 occur independent of *Atoh7*, *Atoh7* function is required to maintain RGC survival and
655 proper targeting of RGC axons to the optic nerve head. Following disruption of both
656 *Atoh7* and *Bax*, we observe only a 20% reduction in the number of RGCs relative to
657 *Bax*-deficient controls. This compares to a greater than 95% reduction in RGC numbers
658 in *Atoh7* mutants relative to wildtype controls. Although RGCs in *Atoh7*^{-/-};*Bax*^{-/-} retinas
659 show severe defects in targeting the optic nerve head, they respond robustly to
660 photoreceptor stimulation. The presence of specified RGCs in the absence of *Atoh7*
661 helps explain long-standing, puzzling observations: 1) 45% of RGCs are not derived
662 from *Atoh7*-expressing progenitors; 2) molecular markers of RGCs are observed at
663 considerably higher levels during early stages of retinal development than in adults in
664 *Atoh7*-deficient retinas; and 3) the marked increase in apoptosis in the ganglion cell
665 layer that occurs in the absence of *Atoh7* (Brzezinski et al., 2012; Feng et al., 2010;

666 Prasov and Glaser, 2012). Previous studies have implicated *Atoh7* as a direct upstream
667 regulator of the essential RGC transcription factors *Brn3a*, *Brn3b*, and *Isl1*. Supporting
668 this, an *Atoh7* hierarchy of RGC determinants, studies in which *Brn3b* and *Isl1* were
669 inserted in place of the *Atoh7* coding sequence observed a complete rescue of normal
670 RGC development (Wu et al., 2015). Our studies, however, indicate that when
671 apoptosis is inhibited, *Brn3a*, *Brn3b*, *Isl1*, and *Rbpms* expression is induced at near
672 normal levels within RGCs in both E14 and adult retinas, regardless of *Atoh7*
673 expression. Therefore, we posit that factor(s) in addition to *Atoh7* activate expression of
674 these genes.

675 The molecular mechanisms by which *Atoh7* controls axonal guidance and cell
676 survival remain unclear. One hypothesis is that RGC axonal guidance and cell survival
677 are linked and that loss of *Atoh7* expression fails to initiate expression of target-derived
678 trophic cues. Because other studies have failed to detect substantial numbers of RGCs
679 in *Atoh7*^{-/-} mice at P0 (Brown et al., 2001; Feng et al., 2010; Prasov and Glaser, 2012;
680 Wang et al., 2001) and target derived neurotrophic factors regulate apoptosis of RGCs
681 between P0 and P12, we feel that *Atoh7*-induced cell survival is functioning at earlier
682 time points than target-derived trophic signals. Additionally, as we did not observe RGC
683 rescue within *Atoh7*^{Cre/Cre};*Bax*^{fl/fl} mice, we suggest that immature RGCs rapidly
684 degenerate as the result of the lack of an *Atoh7*-dependent survival factor. This wave of
685 developmental apoptosis has been observed previously, but the underlying molecular
686 mechanisms are unknown (Farah and Easter, 2005; Frade et al., 1997; Ogilvie et al.,
687 1998; Péquignot et al., 2003; Rodríguez-Gallardo et al., 2005; Strom and Williams,
688 1998; Valenciano et al., 2009). We hypothesize that this unknown factor or factors must
689 be produced by either *Atoh7*-expressing neurogenic RPCs or RGCs derived from these
690 cells.

691 Our data also reveal a marked delay in formation of RGCs from neurogenic
692 RPCs in the absence of *Atoh7*. This is consistent with results from others that in *Atoh7*^{-/-}
693 retina, RGC formation is delayed by at least a day (Le et al., 2006; Prasov and Glaser,
694 2012). When *Atoh7*-dependent RGCs are rescued later in development, as seen in
695 targeted mutants in which *Atoh7* is expressed from the endogenous *Crx* locus, the
696 hyaloid vasculature regression was not rescued, even though a modest rescue of RGC

697 formation is observed (Prasov and Glaser, 2012). In zebrafish retina, consistent with
698 this result, loss of function of *atoh7* in early-stage RPCs disrupts correct targeting of
699 axons in later-born RGCs to the optic nerve (Pittman et al., 2008).

700 Since *Atoh7* was previously thought to be a master transcriptional regulator of
701 RGC specification, strategies aimed at targeted differentiation of RGCs for therapeutic
702 purposes have focused on using forced expression of *Atoh7*. We, however, now
703 appreciate RGC specification to be a far more complicated process. Although ectopic
704 expression of *Atoh7* activates expression of RGC-specific genes in cultured retinal
705 progenitor cells (Yao et al., 2007), induced pluripotent stem cells (Chen et al., 2010),
706 and Müller glia derived retinal stem cells (Song et al., 2015, 2014), it is nonetheless
707 typically not sufficient to drive these cells to become RGCs. This study sheds light on
708 why this may be the case.

709 These findings demonstrate that additional factors act in parallel to *Atoh7* to
710 control RGC specification. While multiple other transcription factors have been reported
711 to regulate RGC specification -- including *Neurod1*, *Sox4*, and *Onecut2* (Jiang et al.,
712 2013; Mao et al., 2013; Sapkota et al., 2014) -- these factors are unable to individually
713 activate expression of *Brn3b* and *Isl1*. In *Atoh7*^{-/-};*Bax*^{-/-} RGCs, however, we observe
714 substantially increased expression of each of these transcription factors (Figure 4E;
715 Supplemental Figure 10), suggesting the possibility that these factors, among others,
716 may compensate for the loss of *Atoh7*. The identity of the non-cell-autonomous cues by
717 which *Atoh7* regulates RGC survival, axon guidance, and hyaloid vasculature
718 regression remain unknown. Further identification of the mechanisms regulating the
719 interplay of intrinsic and extrinsic signals on RGC specification, survival, and maturation
720 will help guide the future design of therapies aimed at maintaining and/or replacing
721 damaged/lost RGCs.

722
723

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733 **Figure Legends**

734

735 **Figure 1.** *Atoh7*-independent development of RGCs. (A-C) We observed a $25.2 \pm 0.9\%$
736 and $21 \pm 3\%$ reduction in RBPMS+ RGC density or Isl1+ ganglion cell layer (GCL) cells
737 when comparing *Atoh7*^{-/-};*Bax*^{-/-} to *Bax*^{-/-} mice. (D,E,F,G,H) Brn3a and Brn3b positive
738 RGC density are only moderately reduced when apoptosis is blocked in *Atoh7*^{-/-};*Bax*^{-/-}
739 mice. (G,H) Brn3a positive RGC numbers are rescued when apoptosis is blocked in all
740 neural retinal progenitor cells, when *Bax*^{lox/lox} is crossed to the *Chx10-Cre* transgene,
741 which is expressed in all RPCs. However, when *Bax* is specifically removed in *Atoh7*-
742 *Cre* knock-in mice, Brn3a RGCs are not rescued. Mean \pm 95% confidence intervals.
743 Statistical significance tested by one-way ANOVA with Tukey's post test for multiple
744 comparisons **** $p < 0.0001$.

745

746 **Figure 2.** *Atoh7* is not required for normal retinal wiring and electrophysiological
747 function. Cells from *Atoh7*^{+/-};*Bax*^{-/-}, and *Atoh7*^{-/-};*Bax*^{-/-} mice responded to light similarly
748 as those from wild type. (A) Two different examples, corresponding to two different sets
749 of RGCs, per genotype of peristimulus time histogram averaged from 120 repetition of
750 1-Hz square wave flash: WT (upper panel), *Atoh7*^{+/-};*Bax*^{-/-} (middle), and *Atoh7*^{-/-};*Bax*^{-/-}
751 (lower panel). (B) Distribution of the spatial receptive field measured using white noise
752 flickering checkerboard: WT (upper, cell count = 92), *Atoh7*^{+/-};*Bax*^{-/-} (middle, cell count =
753 94), and *Atoh7*^{-/-};*Bax*^{-/-} (lower, cell count = 56). (C) the peristimulus time histogram
754 (PSTH) of responses to square-wave flash was calculated using 10-ms bins. Mice
755 assayed at P30. one-way ANOVA, followed by Dunnett's test, $p < 0.05$, between *Atoh7*-
756 ^{-/-};*Bax*^{-/-} and wildtype.

757

758 **Figure 3.** RGC axon guidance and retinal vasculature development requires *Atoh7*-
759 dependent RGCs. (A,D) Smi-32 labels a subset of RGCs and their axons in an adult
760 wildtype retina. In *Atoh7*^{-/-} mice, the Smi-32 positive RGCs have axon guidance deficits.
761 In *Atoh7*^{-/-};*Bax*^{-/-} mice, RGCs have severe axon guidance deficits. Highlighted region (A
762 - *Atoh7*^{-/-};*Bax*^{-/-}) is magnified in (D). (B,C) Using the contralateral pupillary light response
763 as a readout of retina to brain connection allows the appreciation that the severe axon

764 guidance deficits allow for some connection to the brain of the RGCs in the *Atoh7*^{-/-} or
765 *Atoh7*^{-/-};*Bax*^{-/-} retinas. (E) It has been previously reported that the hyaloid vasculature
766 fails to regress in *Atoh7*^{-/-} mice, thought to be due to lack of RGCs, however when the
767 RGC numbers are rescued, in *Atoh7*^{-/-};*Bax*^{-/-} mice, the hyaloid vasculature fails to
768 regress. However, *Atoh7* is not necessary for the hyaloid regression and retinal
769 vasculature development, seen using *Atoh7*^{tTA/tTA};*B&I*^{EE} mice, which was previously
770 seen to rescue all of the *Atoh7* null phenotypes. When *Atoh7* is rescued using the
771 *Crx*>*Atoh7* transgene on the *Atoh7* null background, the optic nerve and 12% of RGCs
772 are rescued (Prasov and Glaser, 2012), but the hyaloid vasculature does not regress.

773
774 **Figure 4.** Single-cell analysis of E14.5 mutant retinas and gene detection in E14
775 retinas. (A,B) UMAP dimension reduction of aggregated E14.5 single cell dataset
776 colored by (A) genotype and (B) annotated cell-type. (C) Proportions of cell types
777 derived from each genotype. (D) Proportions of annotated cell types within each
778 genotype. (E) Heatmap of differentially expressed transcripts across control and *Atoh7*
779 knockout (*Atoh7*^{-/-} or *Atoh7*^{-/-};*Bax*^{-/-}) neurogenic and RGCs. (F) UMAP dimension
780 reduction of cells colored by Scanpy pseudotime values. (G) Density of retinal ganglion
781 cells along pseudotime by genotype. (H) Heatmap displaying differentially expressed
782 transcripts across the interaction of pseudotime and genotype. Cells are ordered by
783 pseudotime within each genotype. (I) Chromogenic *in situ* hybridization detecting RNA
784 transcripts of genes from (H). Insert, depicted by a red dotted line, *Atoh7*^{-/-};*Bax*^{-/-} mice in
785 (H) show robust galanin signal in a region outside the retina, but minimal signal in the
786 retina (J-K). Immunohistochemistry detecting (J) RGC-specific markers: BRN3A and
787 BRN3B and (K) pan-RGC markers: ISL1 and RBPMS in E14 retina from each genotype.
788 Scale bar, 50 μm. Abbreviations: RPCs, retinal progenitor cells; RGCs, retinal ganglion
789 cells.

790
791 **Figure 5.** scCoGAPs analysis of single-cell dataset and RGC population changes in
792 E12.5 retinas show a developmental delay in *Atoh7*^{-/-};*Bax*^{-/-} mutants. (A) Heatmap
793 showing the correlation between scCoGAPS pattern and cellular features. (B) Heatmap
794 of pattern weights within individual cells ordered by pseudotime. Pattern correlations

795 with both pseudotime and each genotype are displayed at the right. (C-E) UMAP
796 embedding of single-cell dataset used for scCoGAPS and colored by (C) celltype or (D)
797 genotype. (E) UMAP embedding of dataset and colored by pattern weights of
798 scCOGAPS patterns 4, 17 and 9, displaying progressive pattern usage across RGC
799 development.

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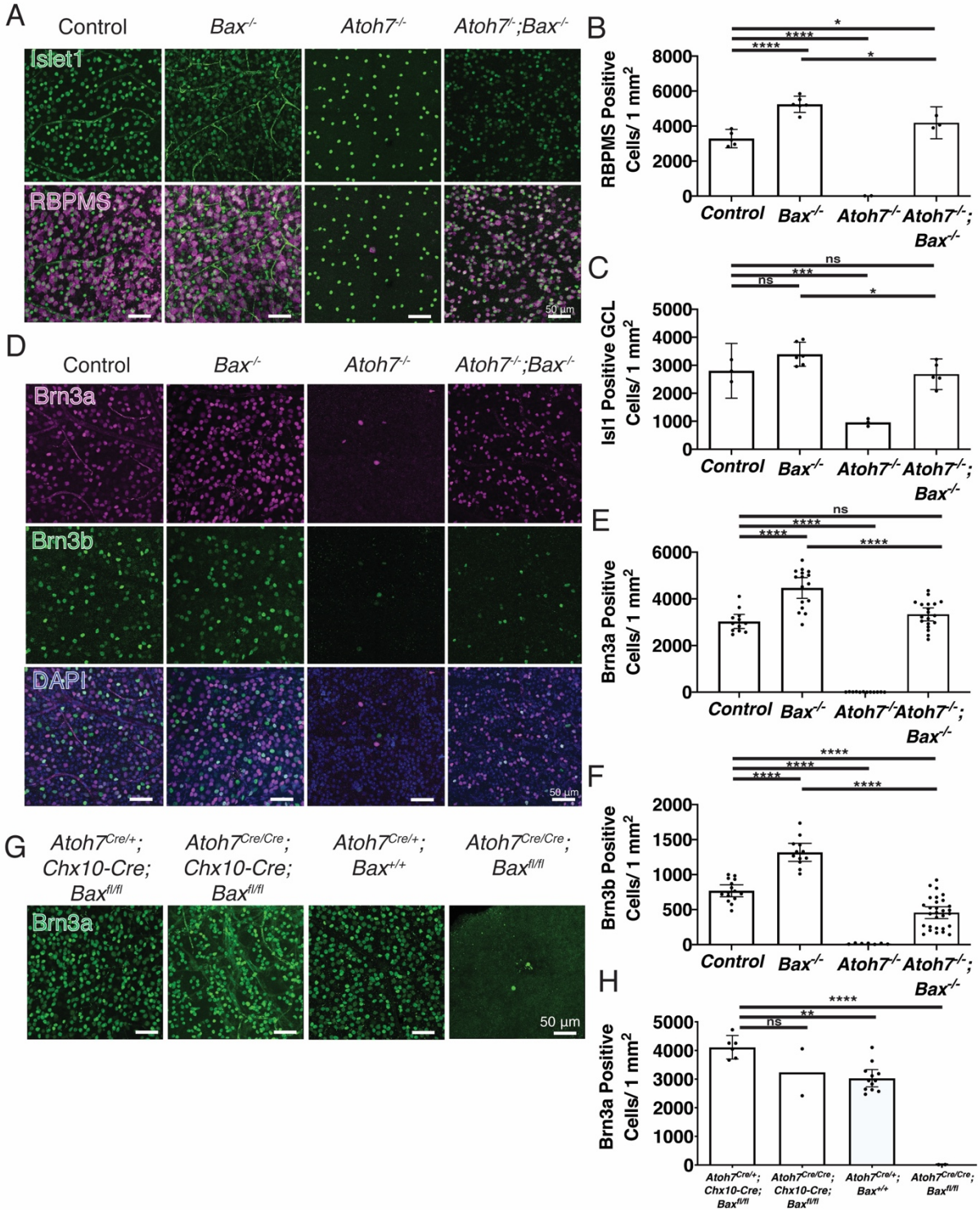


Figure 1. Brodie-Kommit et al. 2020

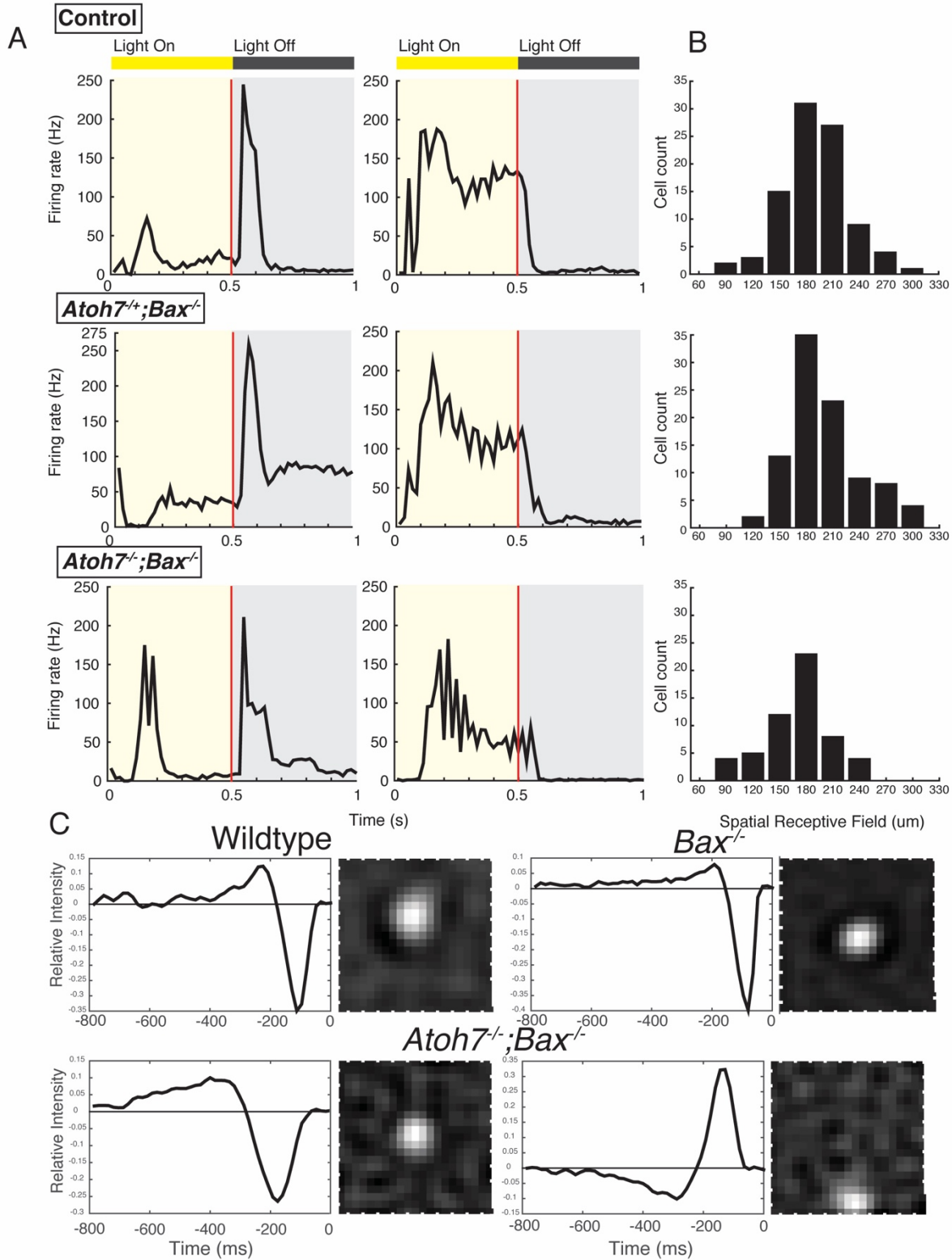


Figure 2. Brodie-Kommit et al. 2020

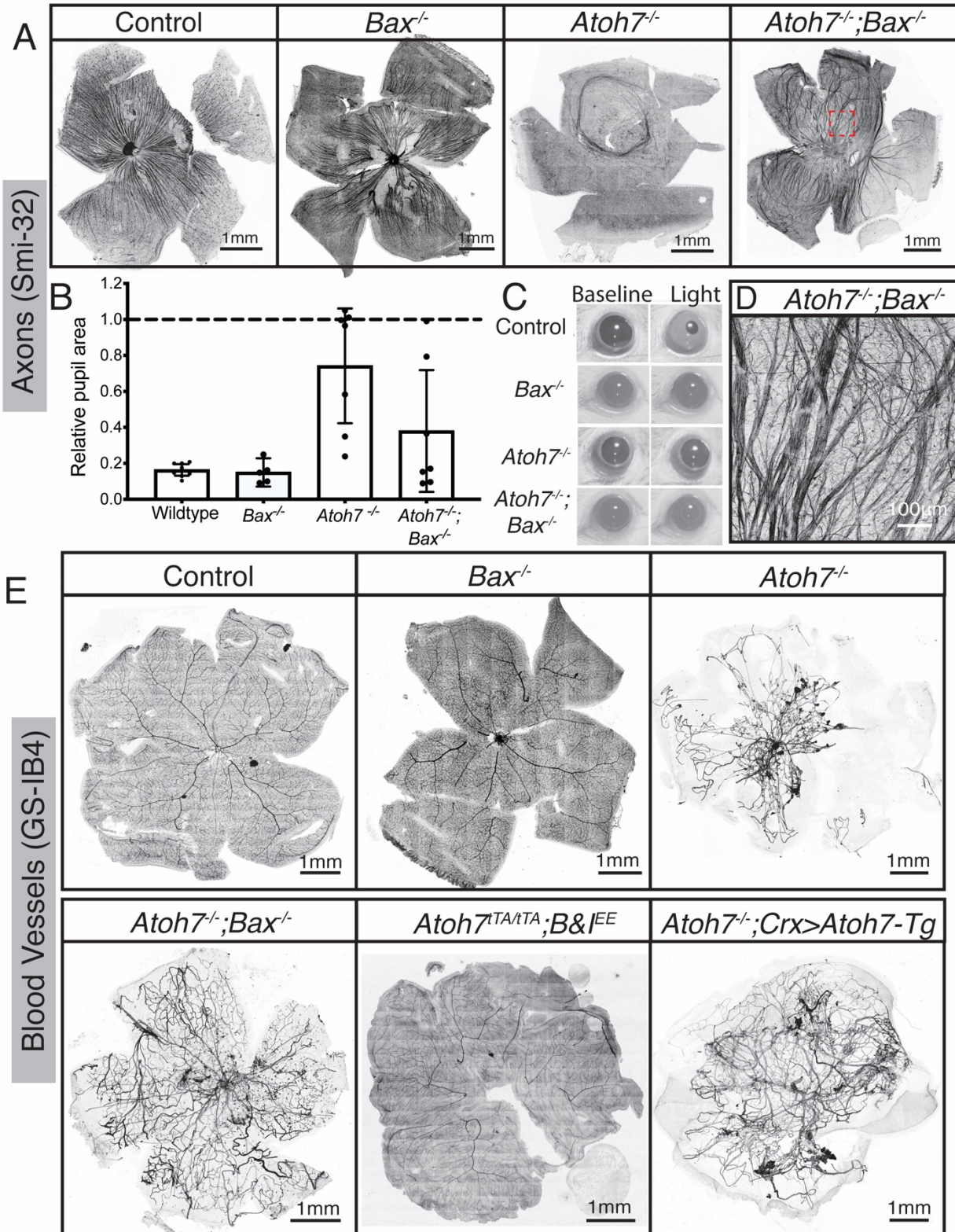


Figure 3. Brodie-Kommit et al. 2020

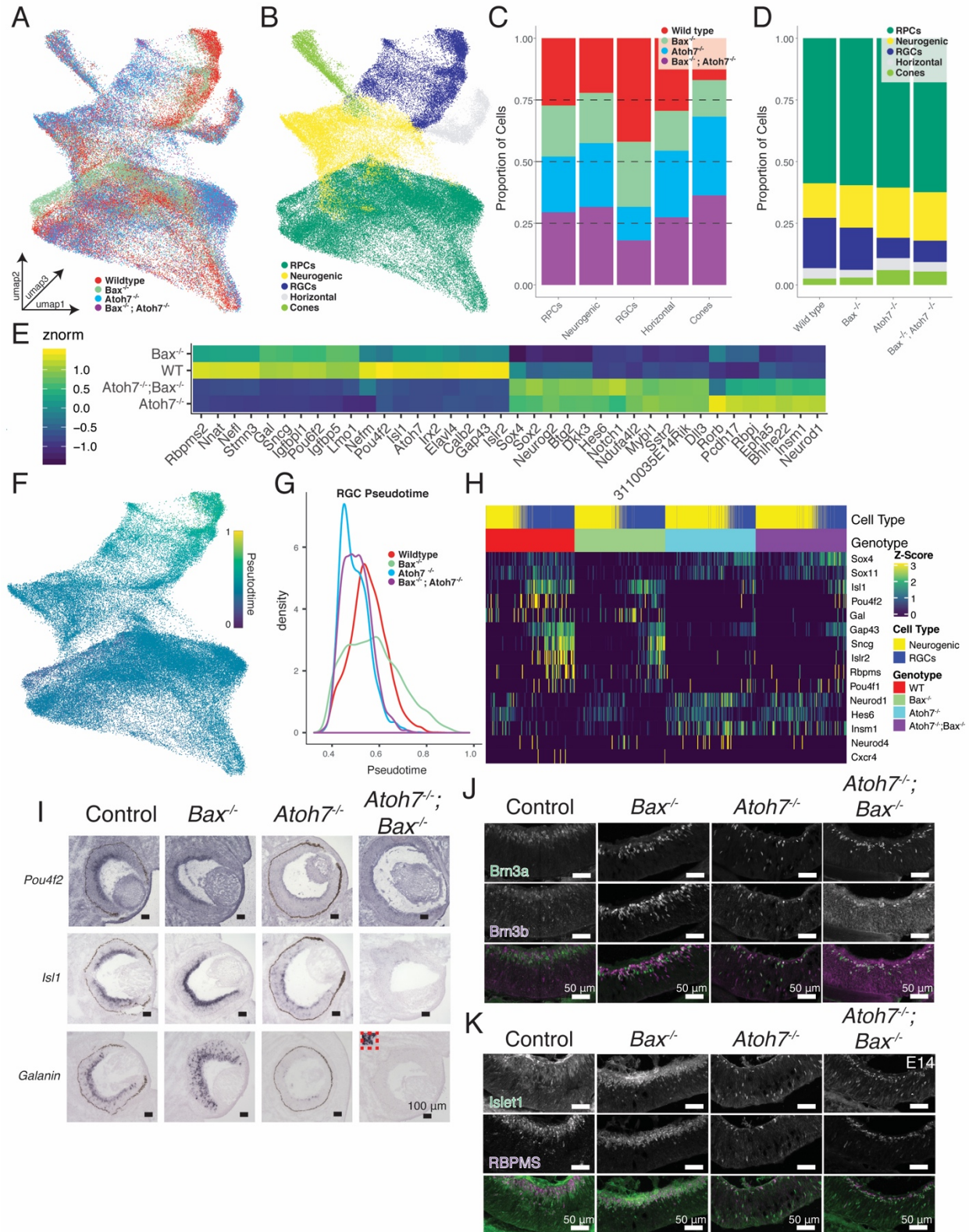


Figure 4. Brodie-Kommit et al. 2020

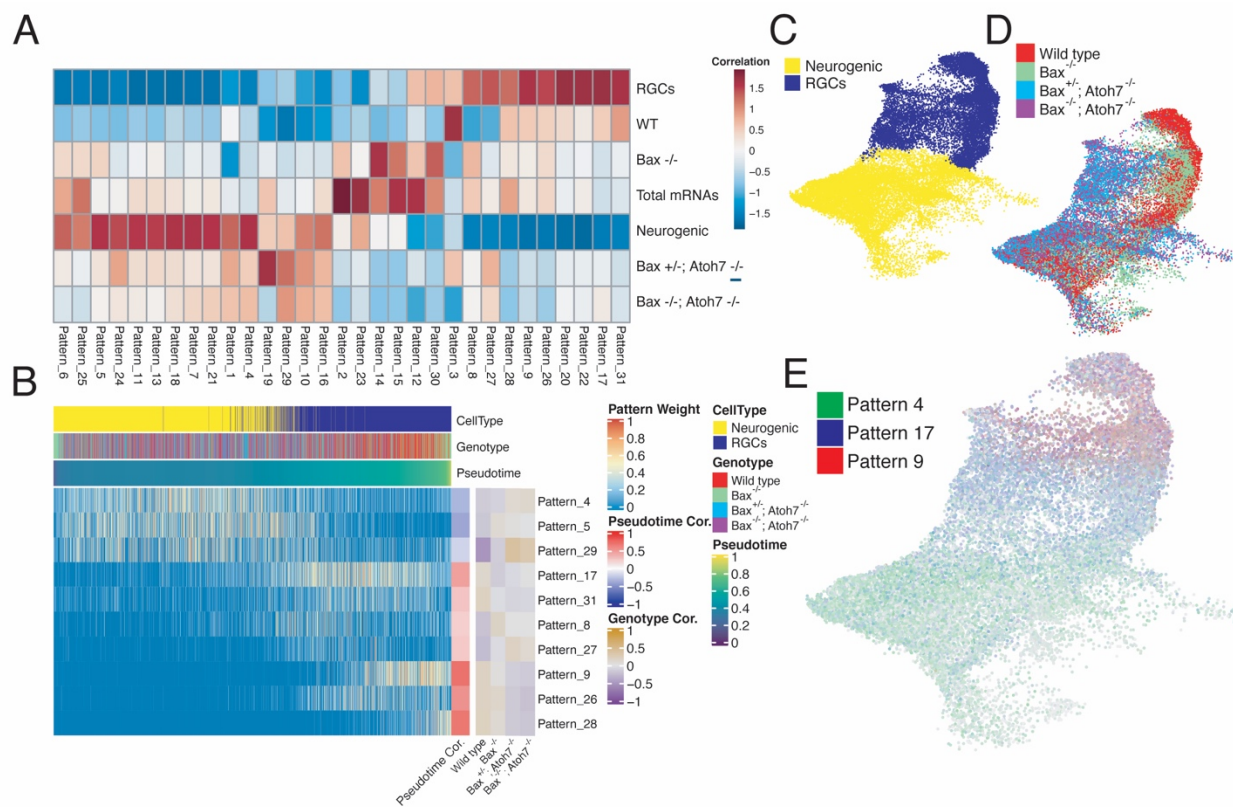


Figure 5. Brodie-Kommit et al. 2020

1142 **Supplemental Methods:**

1143

1144 **Morris water maze**

1145 Vision was tested using a cued Morris water maze (Morris, 1984). A 150cm diameter
1146 pool was filled with water made opaque by mixing in non-toxic white tempera paint. A
1147 platform ("island", not visible from the surface of the water) was placed in one quadrant
1148 with a black and white striped 50 mL conical tube on top as a "flag". Each of six animals
1149 in a group (either *Atoh7^{-/+}*, *Atoh7^{-/-}*, or *Atoh7^{-/-};Bax^{-/-}*) was allowed a 90 second trial in
1150 which to swim and visually locate the island. If the animal found the island, it was
1151 allowed to remain on the island for 60 seconds before being removed from the pool. If
1152 the animal did not locate the island, it was placed at the edge of the island, allowed to
1153 climb on, and remain on the island for 60 seconds to reinforce the island as a safe spot.
1154 Following the first trial, the island was moved to a different quadrant and animals were
1155 tested again. In total, four trials were completed with the island in four different
1156 locations.

1157

1158 **Visual tracking**

1159 Visual acuity was assessed using OptoMotry (Cerebral Mechanics). Animals were
1160 placed on a platform in the viewing chamber and allowed to adapt for 10 minutes prior
1161 to testing. Testing was done for five minutes, during which the position of the animal's
1162 head was monitored to assess visual tracking. Following each tracking event, the
1163 direction and width of the grating was changed, eventually narrowing down to smaller
1164 ranges of acuity.

1165

1166 **X-Gal Staining**

1167 To examine ipRGCs and projections in animals with one allele of *Opn4^{tauLacZ/+}*, adult
1168 animals were transcardially perfused with 10 mL PBS followed by 15 mL of 4% PFA.
1169 Brains were dissected and cryoprotected in 30% sucrose in PBS until the tissue had
1170 sunk in the solution (kept at 4°C) and then frozen in OCT. Cryosections (50 µm) were
1171 mounted on slides and allowed to dry up to 8 hours at room temperature. All X-Gal

1172 staining solutions were prepared according to (Mombaerts et al., 1996). Slides were
1173 rehydrated in Buffer B for 10 minutes, then immersed in Buffer C (with potassium
1174 ferricyanide, potassium ferrocyanide and 100 mg/mL X-Gal in N-N' dimethyl formamide
1175 (DMF)) for 2 days in a dark container. On the third day, fresh Buffer C was added to the
1176 slides which were allowed to sit overnight. Following staining, slides were washed 3
1177 times for 5 minutes in PBS, counterstained in Nuclear FastRed (diluted 1:5 in water) for
1178 10 minutes, washed in 70% ethanol, and mounted in glycerol. Retinas were dissected in
1179 phosphate buffer and placed in X-Gal Buffer B for 10 minutes, then Buffer C for 2 days
1180 at room temperature in a dark container. On the third day, fresh Buffer C was added,
1181 and retinas were stained for an additional day. Following staining, retinas were washed
1182 3 times for 5 minutes in PBS, washed in 70% ethanol, and flat mounted in glycerol.

1183

1184 **CtB sectioning and visualization**

1185 Pulled glass needles were filled via capillary action with roughly 5 μ L of Alexa 488
1186 conjugated Cholera toxin b subunit (CTb; Invitrogen) diluted to 5mg/mL in dH₂O. Filled
1187 needles were affixed to a Harvard Apparatus FIL-190 picospritzer. For injection, adult
1188 animals were anaesthetized with 25ul/g body weight Avertin (1.25% tri-bromo-ethanol
1189 and 2.5% tertiary-amyl alcohol in dH₂O) and tested for alertness via toe pinch. Gentle
1190 pressure was applied behind the eye to raise it, the needles were aimed just above the
1191 skin margin, and CTb was injected in the intraocular space. Animals were allowed to
1192 recover and maintained for three days post-injection to allow the tracer to reach the
1193 distal axons tips. Following the three-day survival period, animals were transcardially
1194 perfused with 15 mL of PBS, followed by 25 mL of cold 4% PFA. Brains were dissected
1195 after perfusion and post-fixed in 4% PFA for 1 hour, then cryoprotected in 30% sucrose.
1196 Brains were frozen in OCT and sectioned at 50 μ m in a cryostat. Sections were dried
1197 overnight, fixed in 4% PFA for 10 min, mounted in VectaShield (Vector Labs)+1.5
1198 mg/mL DAPI and imaged on a Zeiss Imager M1 upright epifluorescence microscope
1199 (AxioVision software).

1200 **Supplemental Figure Legends**

1201

1202 **Supplemental Table 1.** 230 differentially expressed transcripts (q-value < 1e-300)
1203 between control (control and *Bax*^{-/-}) versus *Atoh7*-deficient (*Atoh7*^{-/-}, or *Atoh7*^{-/-};*Bax*^{-/-})
1204 neurogenic and retinal ganglion cells.

1205

1206 **Supplemental Table 2.** Differentially expressed transcripts (q-value < 1e-5) across the
1207 interaction of pseudotime and genotype.

1208

1209 **Supplemental Figure 1.** (A,B) Whole retinal dissection showing the presence of Brn3a
1210 and Brn3b positive RGCs respectively. Notice the orientation of the retinas as marked
1211 by the orientation rose. Highlighted regions (A,B) are magnified in (A',B'). (C,D,E,F)
1212 IpRGCs are partially rescued by preventing apoptosis. (C,D) To determine if ipRGCs
1213 were undergoing apoptosis in the *Atoh7*^{-/-}, ipRGC numbers were examined in *Atoh7*^{-/-}
1214 and *Atoh7*^{-/-};*Bax*^{-/-}. ipRGCs in the *Atoh7*^{-/-};*Bax*^{-/-} were rescued to 60% of wild type levels.
1215 *Bax*^{-/-} showed a 202±9% increase in ipRGCs compared to controls, similar to what has
1216 been reported for other RGCs in the *Bax*^{-/-} knockout. (E,F) To determine if ipRGCs were
1217 generated in development in *Atoh7*^{-/-} mice, I examined E16 and P0 timepoints using the
1218 *Opn4*^{tauLacZ} marker. At both E16 and P0, a 90% deficit of ipRGCs was observed. Mean ±
1219 95% confidence intervals. Statistical significance tested by one-way ANOVA with
1220 Tukey's post test for multiple comparisons ** p=0.0052, **** p < 0.0001.

1221

1222 **Supplemental Figure 2.** *Atoh7*^{Cre};*R26*^{tdTomato} lineage labeling in detail. (A,B) A similar
1223 percent of RGCs are of the *Atoh7* lineage in both wildtype and *Atoh7*^{-/-};*Bax*^{-/-} mice.
1224 Yellow arrows highlight Brn3a+ and *Atoh7*-expressing RPC-derived RGCs while blue
1225 arrows highlight non-Brn3a+ cells in the GCL (ganglion cell layer). (C)
1226 *Atoh7*^{Cre};*R26*^{tdTomato} labels cells of most cell types in the retina in all genotypes
1227 examined. Abbreviations: GCL, INL (inner nuclear layer), ONL (outer nuclear layer).

1228

1229 **Supplemental Figure 3.** During early development, E12.5, *Atoh7*^{-/-};*Bax*^{-/-} mice have
1230 both fewer Brn3a positive RGCs and mature RGCs. (A) Pax2 antibody staining was

1231 used to help find the central retina to make sure the earliest born RGCs in the central
1232 retina were fully sampled. (B) Quantification of the retinal images with the black bars
1233 representing density and the red bar plotted on the right side representing the percent of
1234 mature RGCs. Mean \pm 95% confidence intervals. Statistical Significance tested by
1235 unpaired t test ** $p = 0.0099$ **** $p < 0.0001$. Abbreviations: RGCs, retinal ganglion
1236 cells; GCL, ganglion cell layer.

1237
1238 **Supplemental Figure 4.** Peristimulus time histogram averaged from 120 repetition of 1-
1239 Hz square wave flash of every RGC recorded from in *Atoh7^{-/-};Bax^{-/-}* mice. The red line
1240 represents the transition from Light on to Light off in the square wave light flash.

1241
1242 **Supplemental Figure 5.** Similar results seen with Tuj1 (A), NFM, and NFH (B)
1243 immunostaining as seen with Smi-32 staining. (C) More examples of orientation
1244 preserved dissection of whole retinas of all four genotypes and stained with an antibody
1245 to Smi-32, notice the orientation of the retinas as marked by the orientation rose.

1246
1247 **Supplemental Figure 6.** RGCs in *Atoh7^{-/-};Bax^{-/-}* mice do not sustain visual tasks. In the
1248 virtual optometer, animals visually track a moving grating of varied widths. (A) Only wild
1249 type animals were able to track gratings with an acuity of 0.4 cycles/degree. In the cued
1250 Morris Water Maze (B) animals locate a marked platform to escape the water. In four
1251 successive trials, most wild type animals learned to locate the platform relatively quickly.
1252 (C) ipRGC projections do not reach the brain in *Atoh7^{-/-};Bax^{-/-}* mice. The projections of
1253 ipRGCs were genetically labeled with the tauLacZ marker (depicted here as *Opn4^{+/-}*) in
1254 the *Atoh7^{-/-};Bax^{-/-}* background. Robust innervation is revealed by X-Gal staining in the
1255 control while the *Atoh7^{-/-};Bax^{-/-}* mice do not show innervation (target nuclei outlined in
1256 red). (n=3, scale bars = 100mm). (D) Fluorescently conjugated Cholera toxin b labels
1257 RGC projections. Non-image forming (SCN, OPN) and image-forming targets (LGN,
1258 SC) do not receive retinal innervation in *Atoh7^{-/-}* or *Atoh7^{-/-};Bax^{-/-}* mice. Yellow outlines
1259 depict targets; All scale bars 100mm, n=3 per genotype. Mean \pm 95% confidence
1260 intervals. Statistical significance tested by one-way ANOVA with Tukey's post test for
1261 multiple comparisons * $p=0.0386$, **** $p < 0.0001$. Abbreviations: SCN: Suprachiasmatic

1262 nucleus, OPN: Olivary Pretectal Nucleus, LGN: Lateral geniculate nucleus, SC:
1263 Superior colliculus.

1264

1265 **Supplemental Figure 7.** Traces of Pupillary light reflex (PLR) of individual mice before
1266 and after light stimulus for contralateral (A) and ipsilateral (D) recordings. *Bax*^{-/-} and
1267 *Atoh7*^{-/-};*Bax*^{-/-} mice lack retinal pigmentation therefore their pupils are outlined for clarity.
1268 Analysis of the relative pupil area of all of the PLR responses between genotypes for
1269 contralateral (B) and ipsilateral (C). Mean \pm 95% confidence intervals.

1270

1271 **Supplemental Figure 8.** (A) Table of scRNA-seq statistics for each sample. (B-C)
1272 Boxplots of the number of (B) Expressed Genes and (C) Unique molecular identifier
1273 (UMI) or transcript counts within each cell. (D) UMAP dimension reduction of the
1274 aggregate single cell datasets with individual cells colored by originating sample
1275 identity. (E) UMAP dimension reductions displaying the subset of cells corresponding to
1276 each genotype (top) and the cell type annotations (bottom) of the corresponding cells.
1277 (F) Cell type annotations of cells within each scRNA-seq sample. (G) Heatmap of
1278 known cell type markers within annotated cell types.

1279

1280 **Supplemental Figure 9.** (A) Dotplot of a subset of marker genes used to determine cell
1281 type annotations of clusters within UMAP dimension reduction space. Color of the circle
1282 corresponds to the log() of the mean expression of individual transcripts within cells of
1283 each cluster. Size of the circles corresponds to the percentage of cells within the cluster
1284 that had at least one transcript detected. (B) Heatmap of the cell type enrichment of
1285 *Atoh7*-dependent, differentially expressed transcripts across wildtype mouse retinal
1286 development (E11-P14) as assayed by Clark et al., 2019.

1287

1288 **Supplemental Figure 10.** Pseudotime heatmap of top 10 weighted genes of
1289 scCoGAPS Patterns. Cells are ordered by pseudotime.

1290

1291 **Supplemental Figure 11.** Pseudotime heatmap of previously published RGC subtype
1292 markers and their correlation to pseudotime.

1293

1294 **Supplemental Figure 12.** (A) Immunohistochemistry images of the RGC layer stained
1295 for Brn3a and RBPMS in Galanin heterozygous and knockout retinas. (B) Cell counts of
1296 RGCs indicate no difference in RGC number in P8 galanin-deficient retinas.

1297

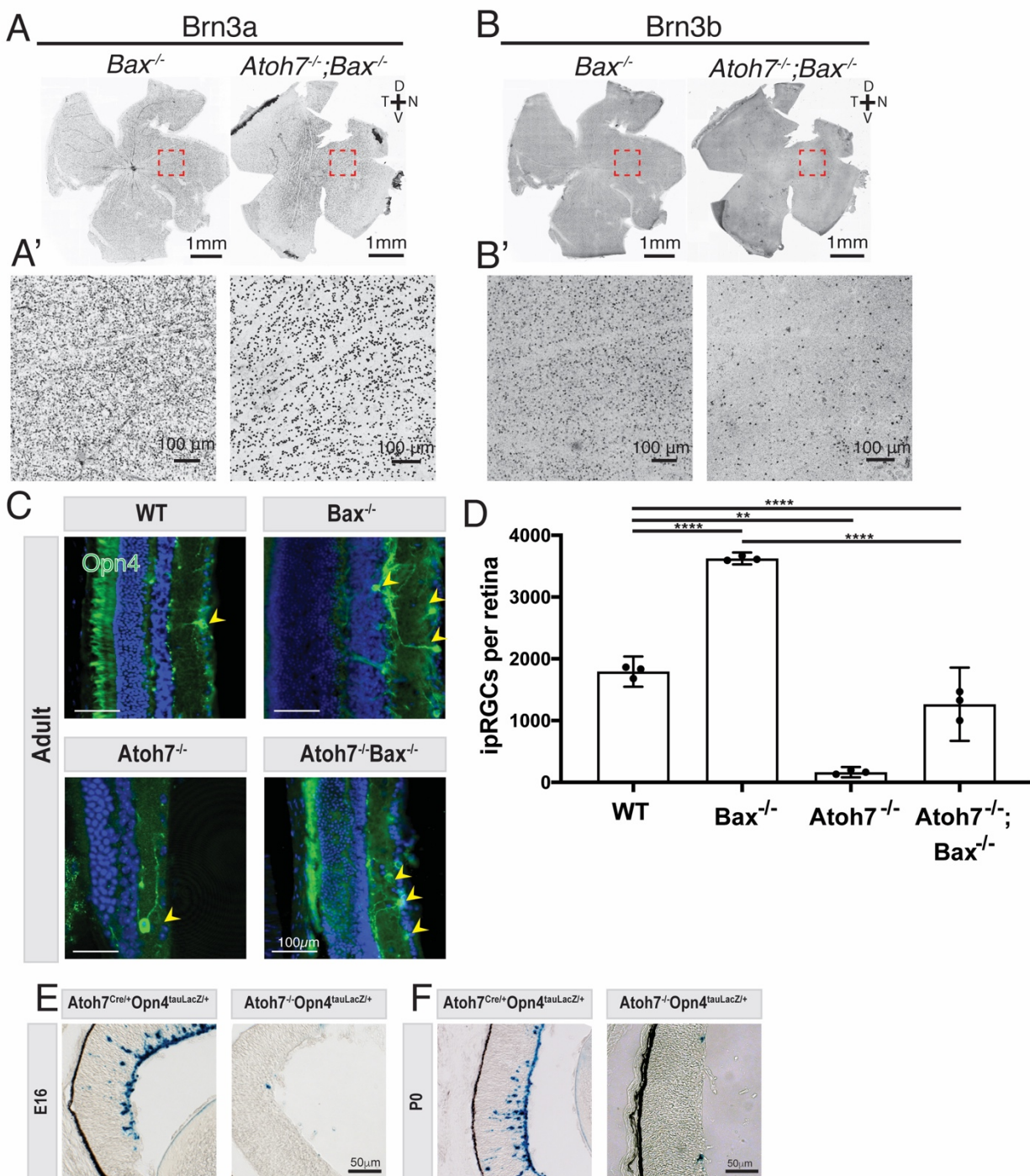
1298 **Supplemental References**

1299

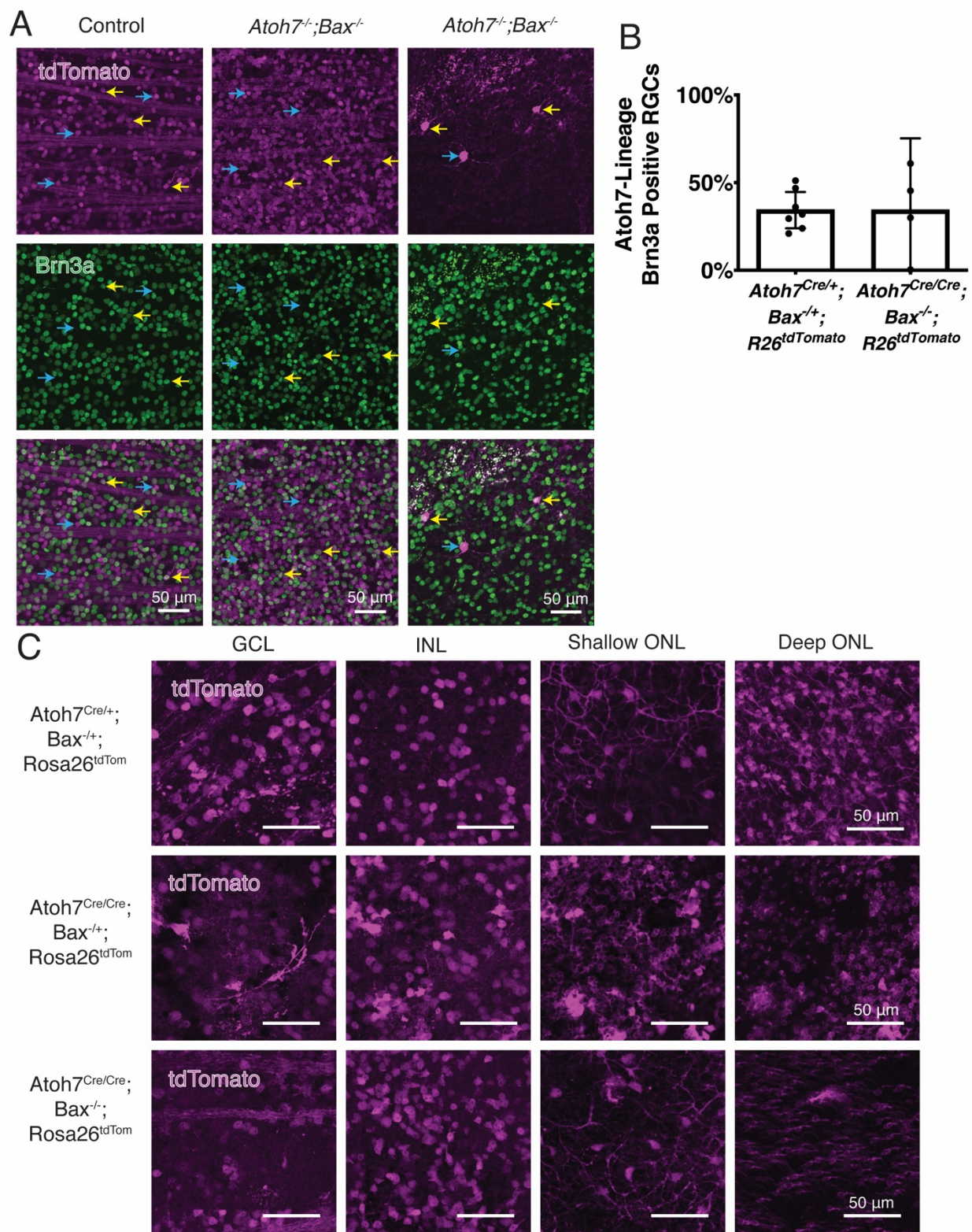
1300 Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M,
1301 Edmondson J, Axel R. 1996. Visualizing an Olfactory Sensory Map. *Cell* 87:675–
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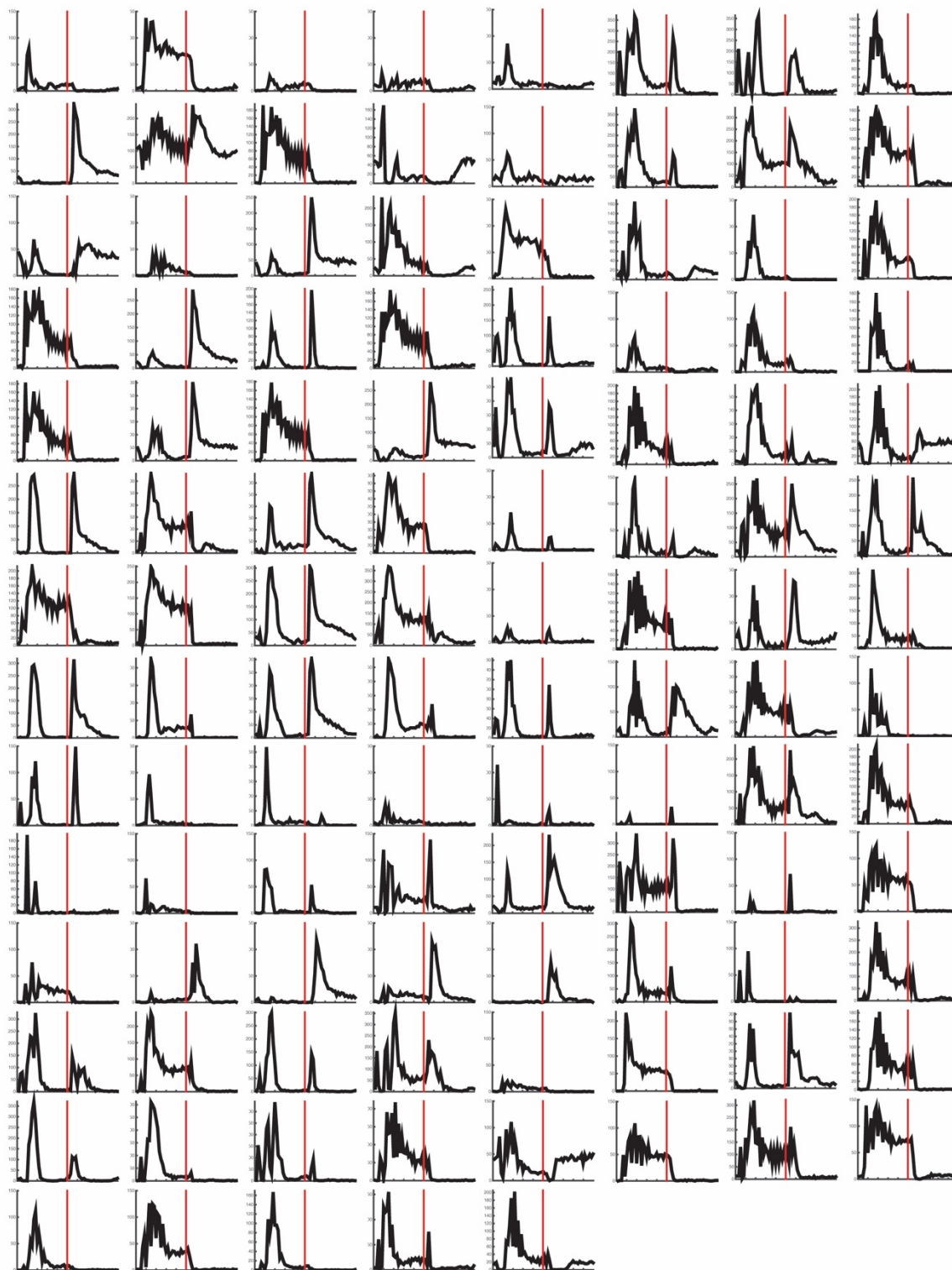
1304 Morris R. 1984. Developments of a water-maze procedure for studying spatial
1305 learning in the rat. *J Neurosci Meth* 11:47–60. doi:10.1016/0165-0270(84)90007-
1306 4



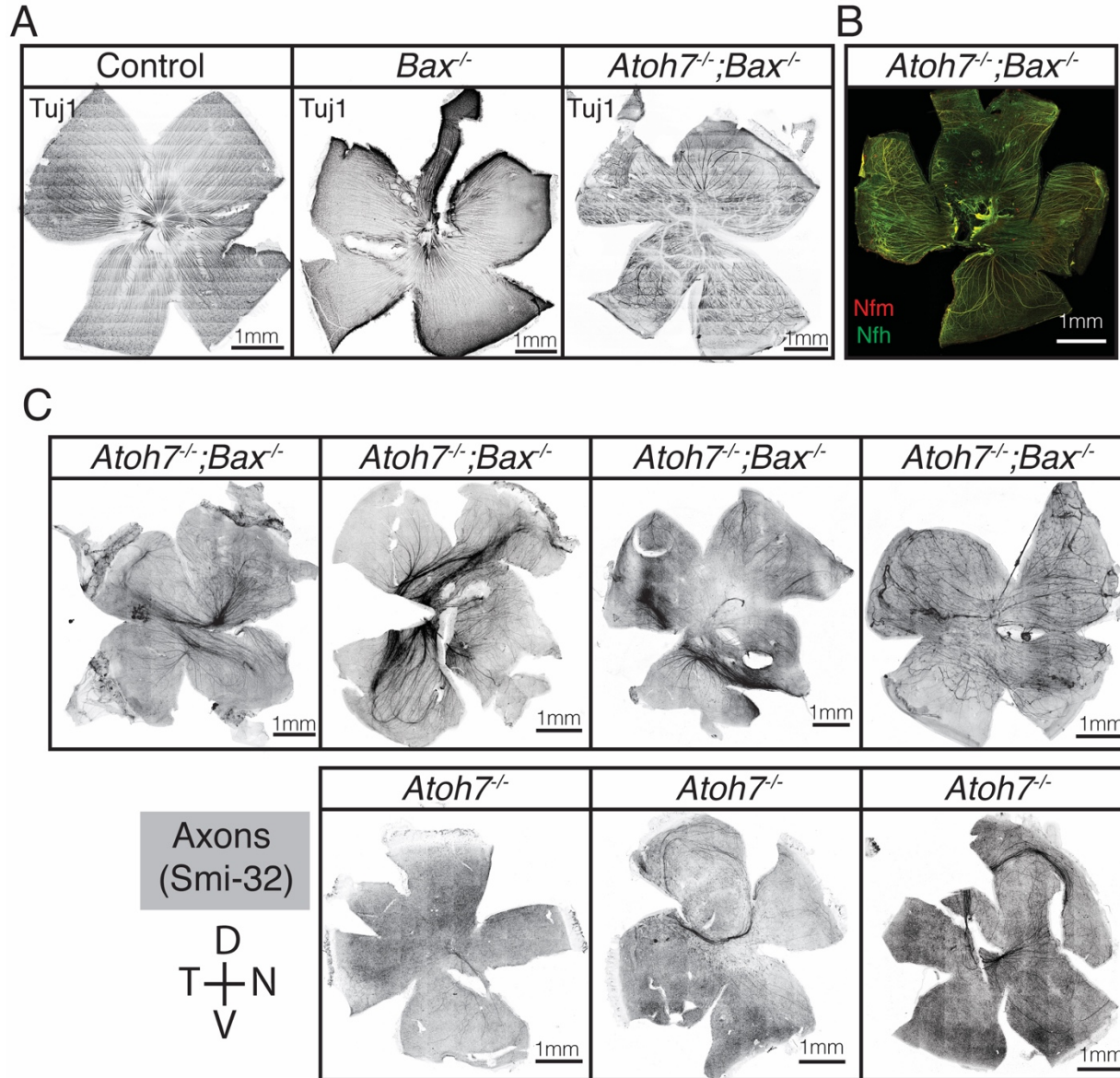
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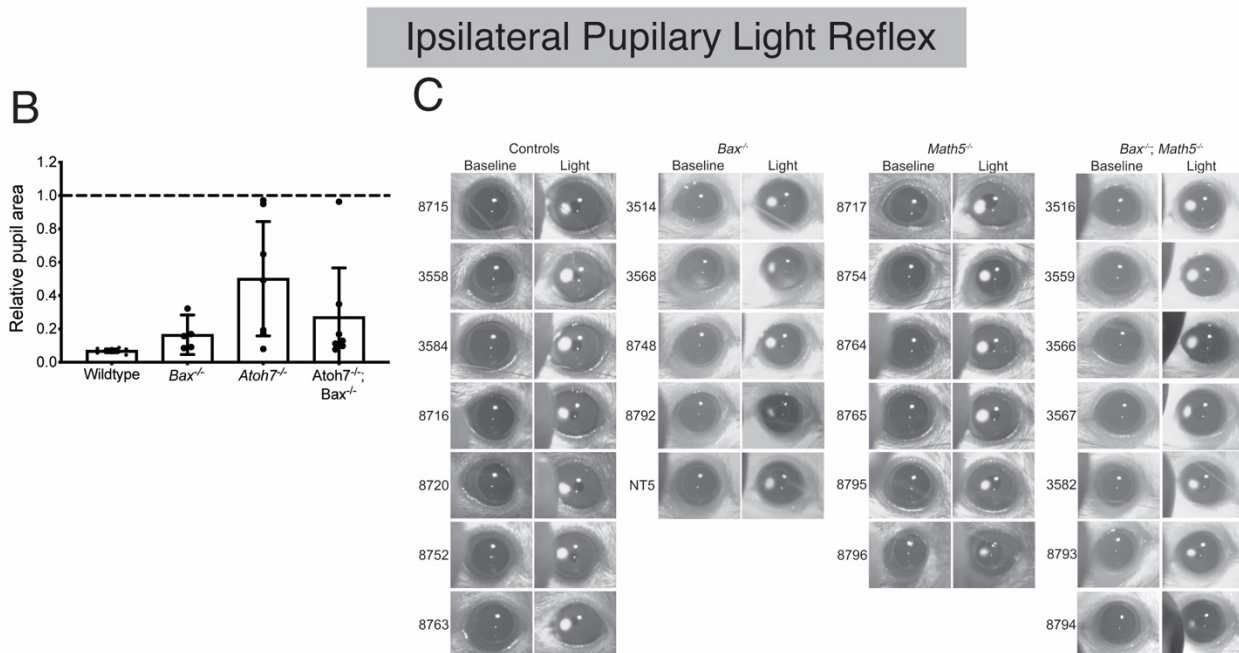
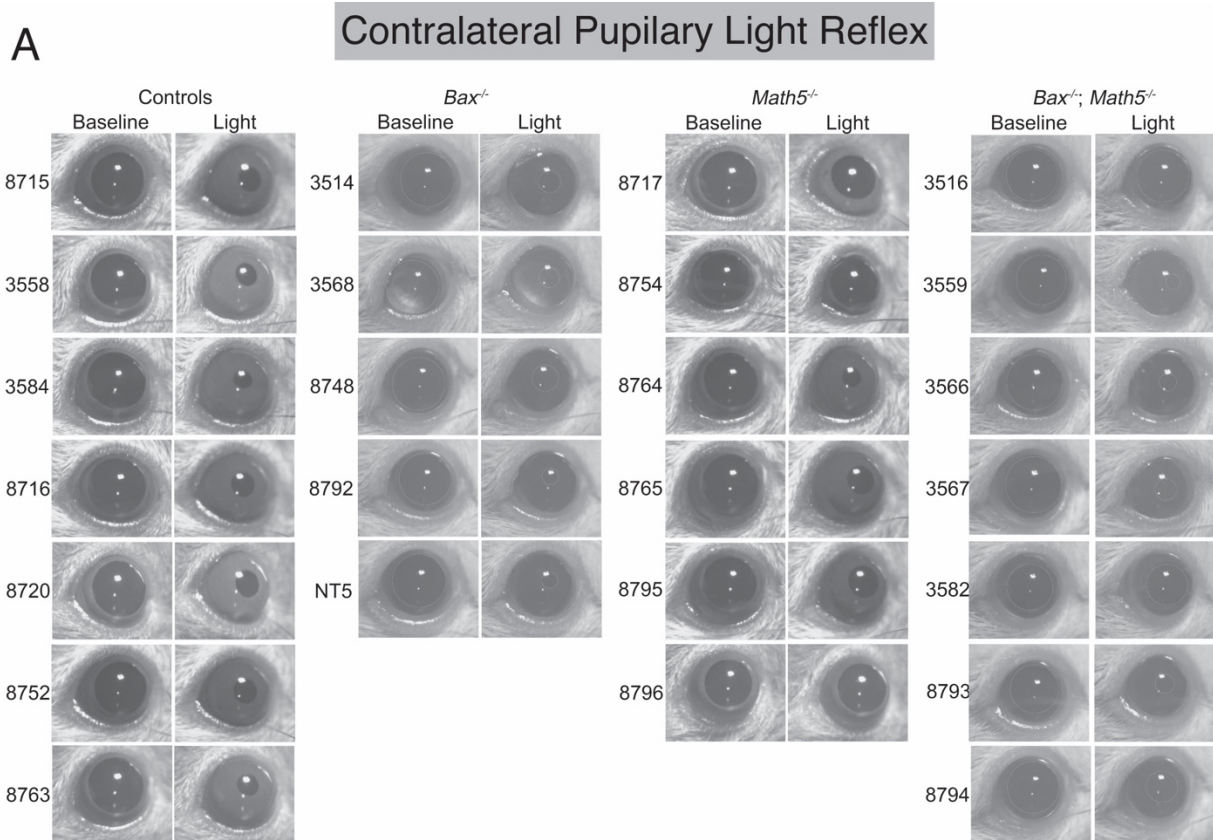
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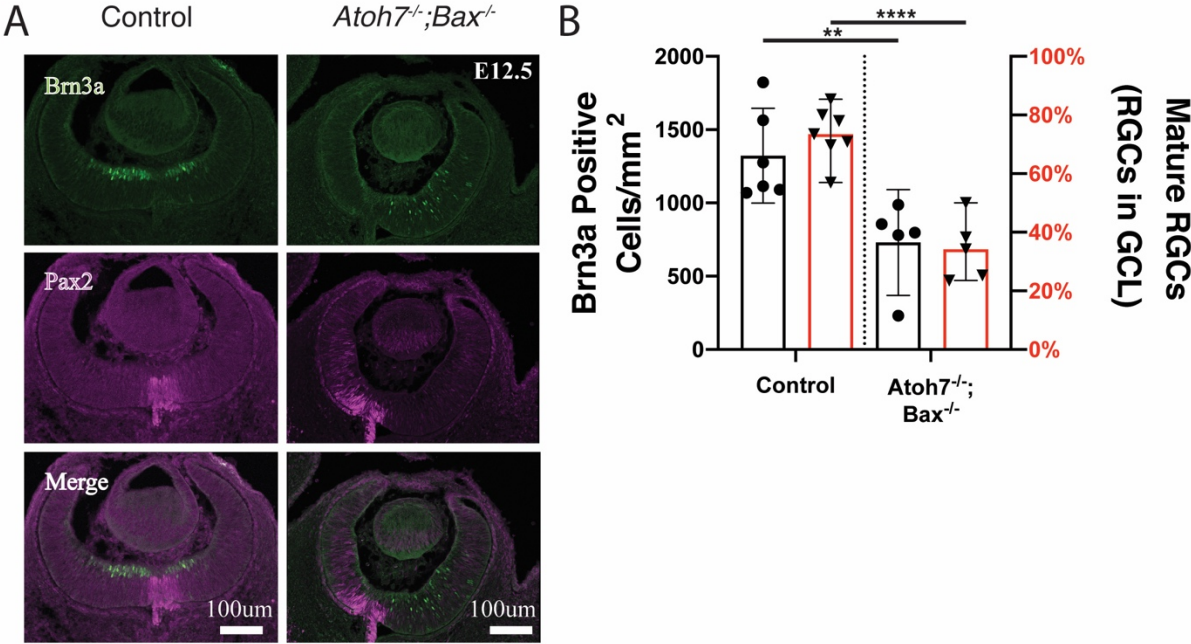
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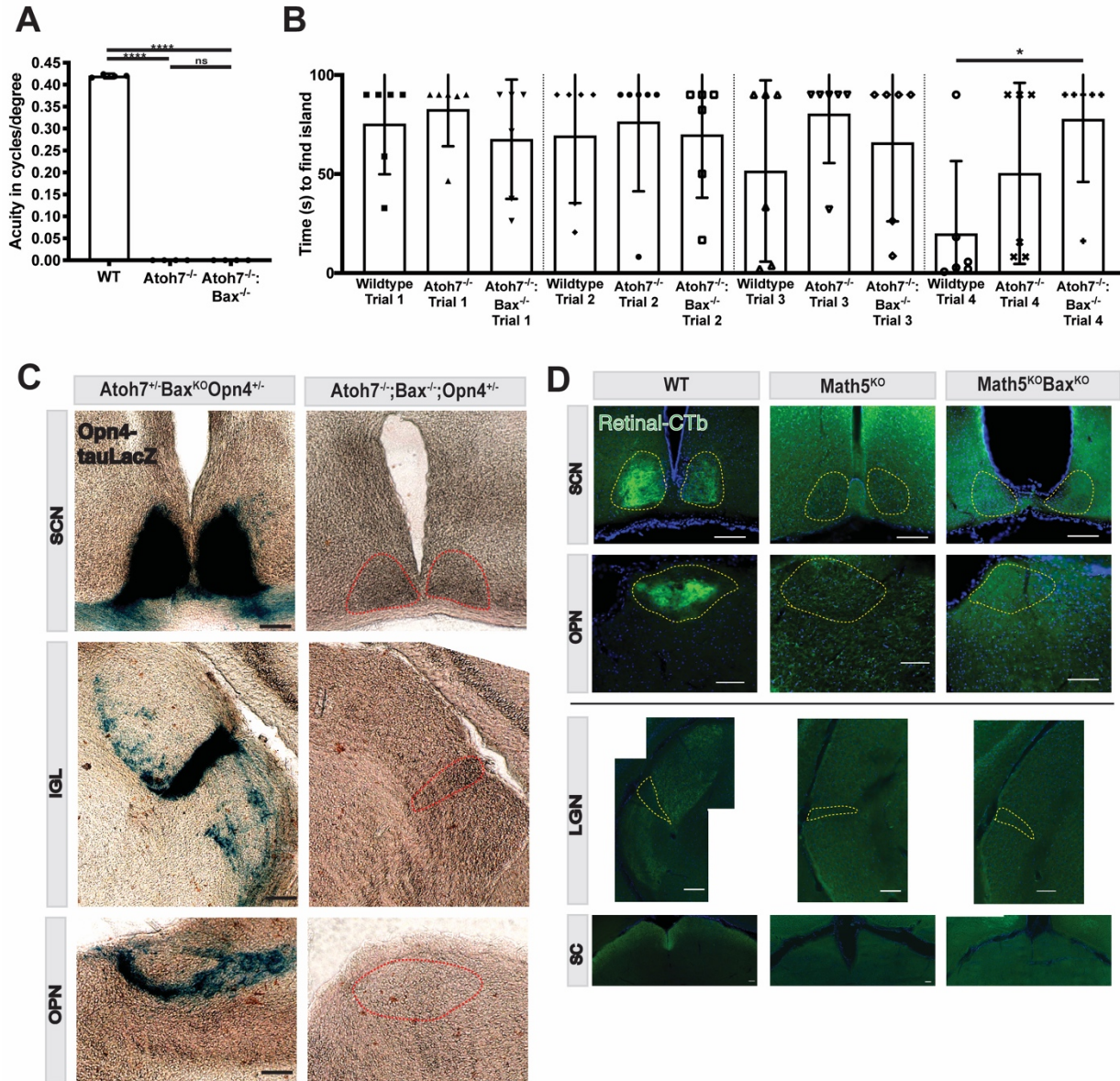
Supplemental Figure 4. Brodie-Kommit et al. 2020



Supplemental Figure 5. Brodie-Kommit et al. 2020



Supplemental Figure 6. Brodie-Kommit et al. 2020

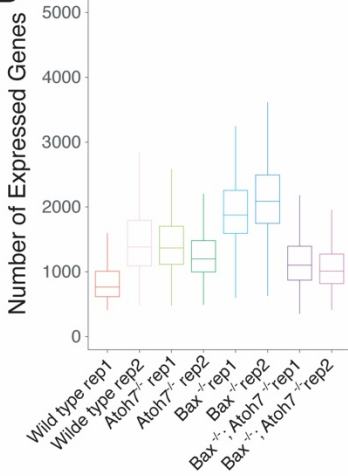


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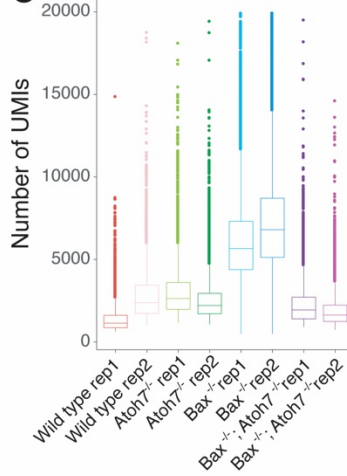
A

Sample	# Cells	Mean UMI	Mean Genes	Age	Genotype	Source
WT_rep1	15582	1392	871	E14	CD1 Wild-type	(Clark <i>et al.</i> , 2019; <i>Neuron</i>)
WT_rep2	10496	2789	1491	E14	CD1 Wild-type	(Clark <i>et al.</i> , 2019; <i>Neuron</i>)
Bax_Het_Atoh7_Null_rep1	9622	3029	1465	E14	Bax +/-; Atoh7 -/-	This Study
Bax_Het_Atoh7_Null_rep1	11345	2547	1299	E14	Bax +/-; Atoh7 -/-	This Study
Bax_Null_rep1	10268	6144	1951	E14	Bax -/-; Atoh7 +/+	This Study
Bax_Null_rep2	9269	7185	2130	E14	Bax -/-; Atoh7 +/+	This Study
Bax_Null_Atoh7_Null_rep1	14730	2269	1193	E14	Bax -/-; Atoh7 -/-	This Study
Bax_Null_Atoh7_Null_rep2	11816	1901	1098	E14	Bax -/-; Atoh7 -/-	This Study

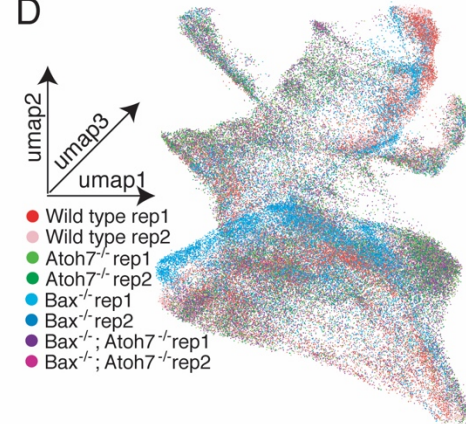
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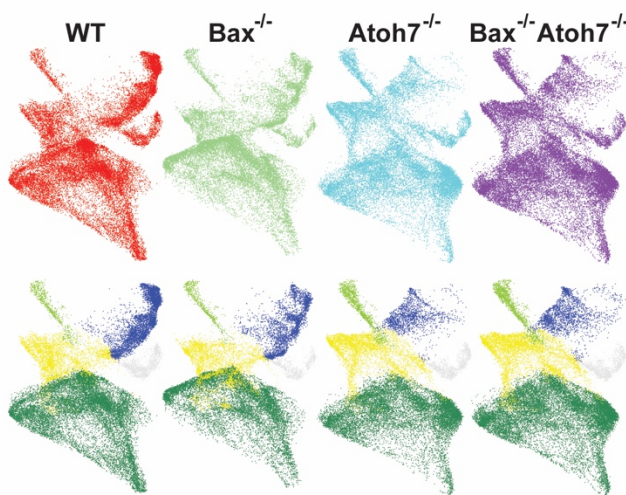
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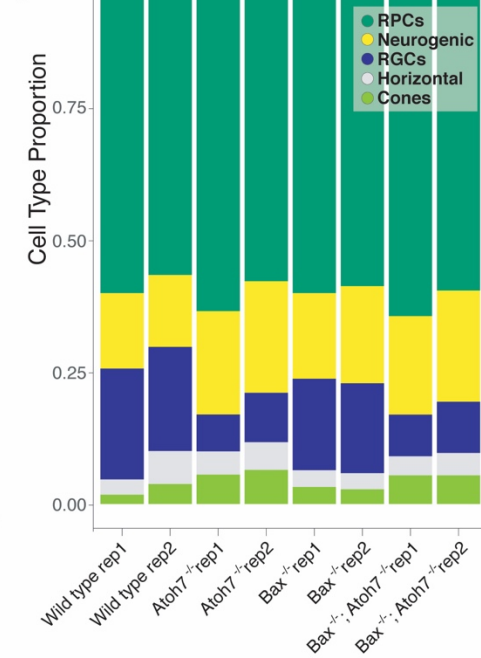
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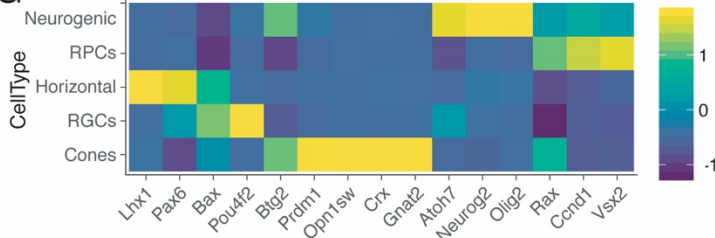
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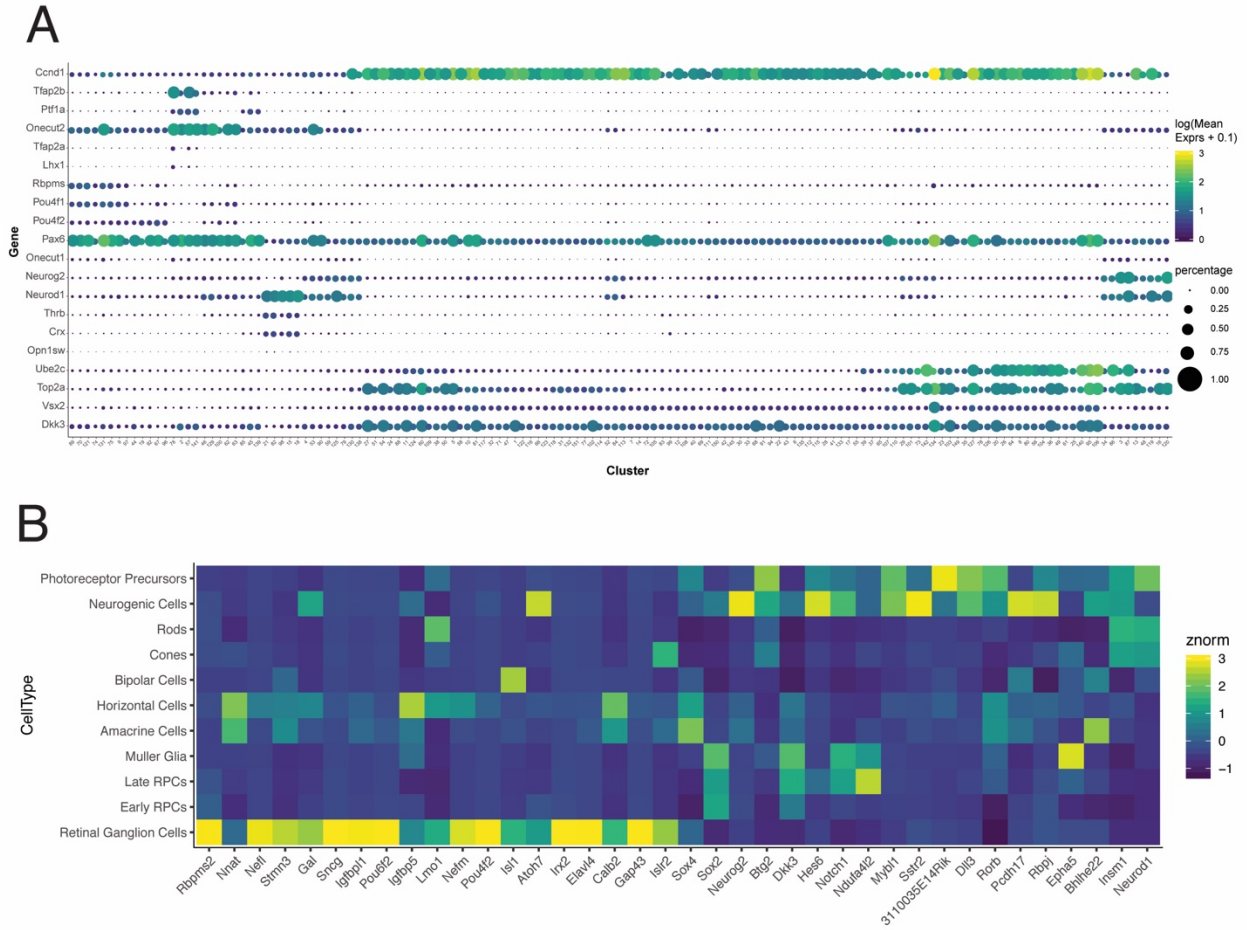
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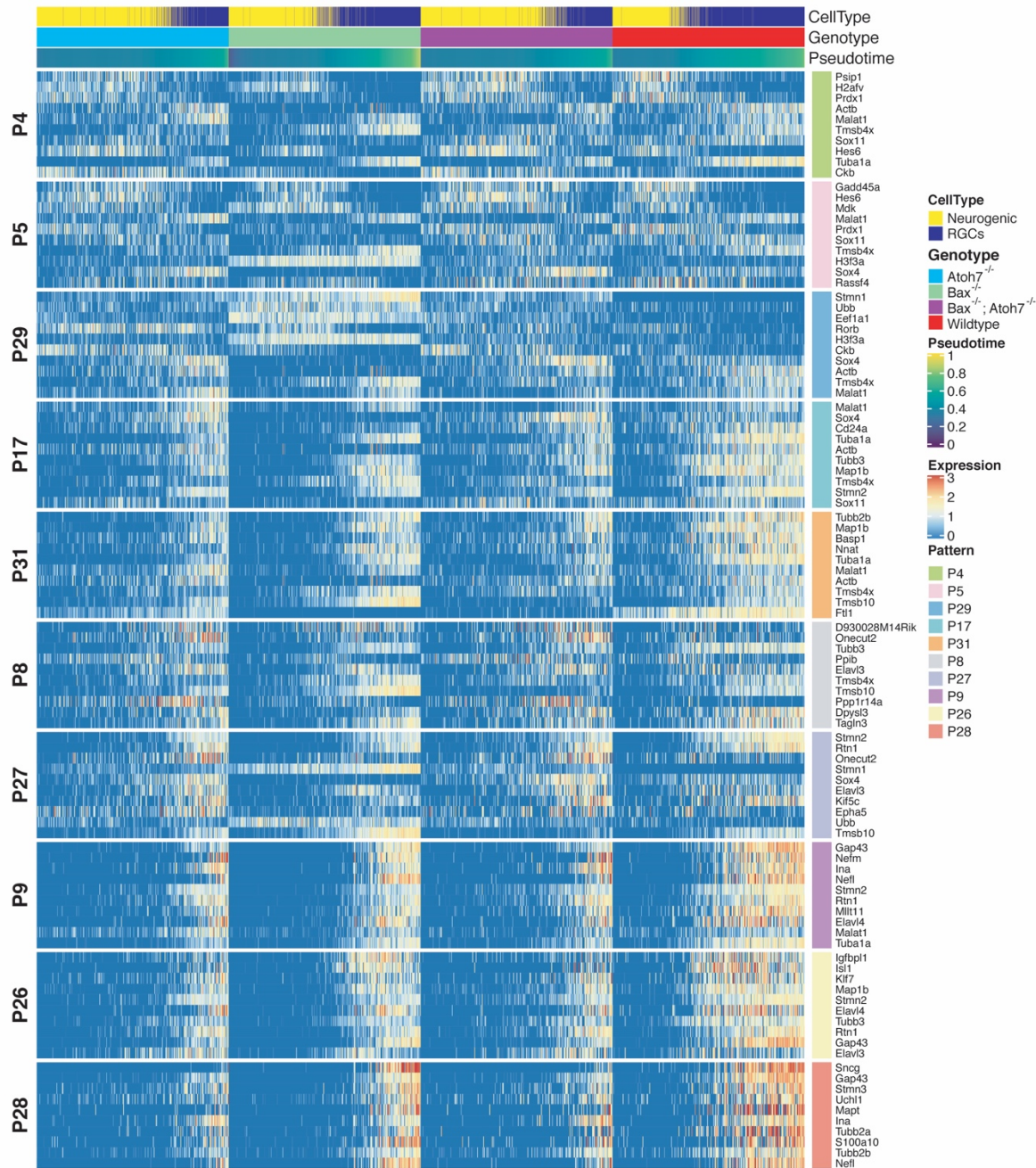
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Supplemental Figure 8. Brodie-Kommit et al. 2020

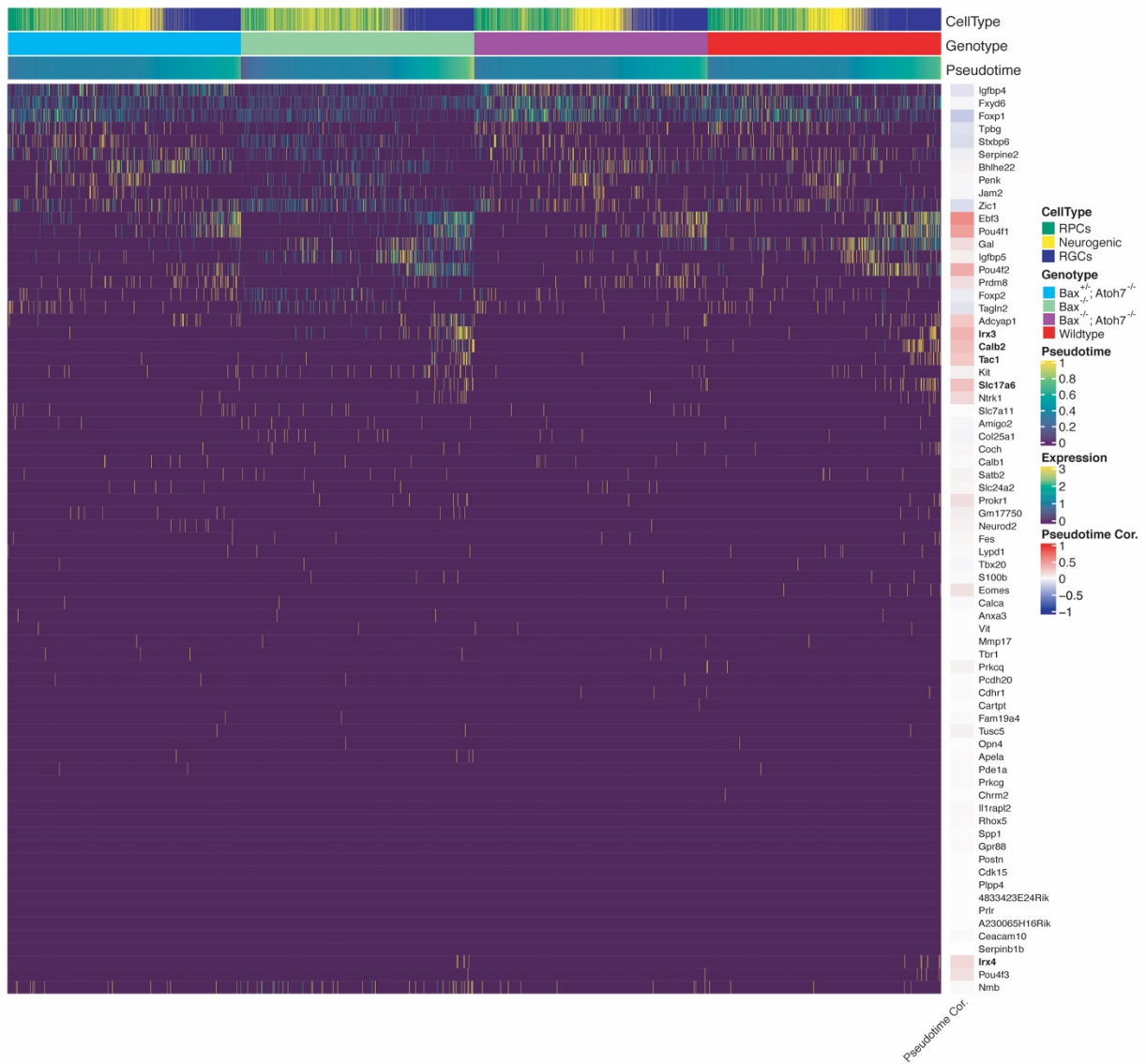


Supplemental Figure 9. Brodie-Kommit et al. 2020

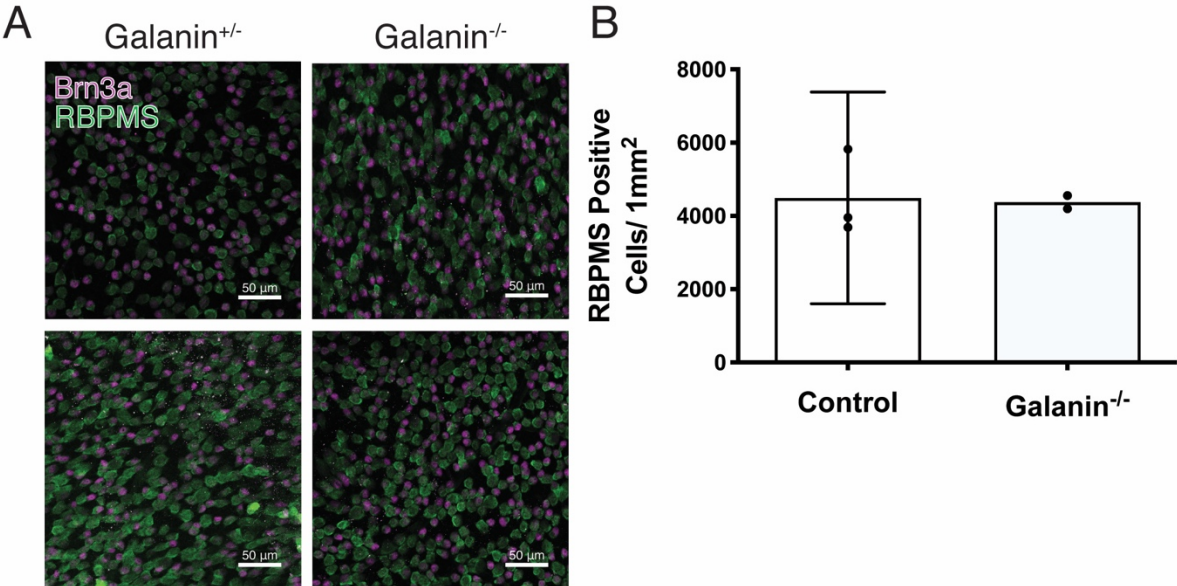


Supplemental Figure 10. Brodie-Kommit et al. 2020

A



Supplemental Figure 11. Brodie-Kommit et al. 2020



Supplemental Figure 12. Brodie-Kommit et al. 2020