

1 **Wnt1-Cre mediated deletion of BMP7 suggests a role**
2 **for neural crest-derived BMP7 in retina development**
3 **and function**

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17

18 **Abstract**

19 Neural crest (NC) contributes to various structures of the eye including cornea, ciliary
20 body and retina. The association of NC-derived cells with hyaloid vessels in the form of
21 pericytes is established. Similarly, persistence of NC-derived cells in the inner retina
22 layer of the mature retina has been suggested. To date, no specific function has been
23 attributed to them. NC-derived Bone morphogenetic protein 7 (BMP7) controls
24 neurogenic properties in the brain and regulates glia differentiation. Here, we assessed
25 the role of NC-derived BMP7 in the adult retina.
26 BMP7 expression was determined using *Bmp7*LacZ reporter mice. *BMP7* was
27 expressed in GCL, IPL, OPL, and photoreceptors in P0, P14 and P30 retinas. Lineage
28 tracing confirmed the presence of NC-derived cells in the GCL, INL, and ONL. Some
29 but not all cells associated with vasculature. To test the function of NC-derived *Bmp7*,
30 *Bmp7^{fl/fl}Wnt1cre* (*Bmp7^{ncKO}*) mice were assessed by histological and functional methods.
31 Loss of NC-derived cells in the GCL and INL and mild structural abnormalities were
32 observed in the *Bmp7^{ncKO}* retina. Electroretinography revealed reduced a wave under
33 photopic conditions and b wave under both scotopic and photopic conditions. The
34 neuronal circuitry in the inner retina appeared affected, evidenced by decreased
35 Calbindin in the GCL, IPL and INL. In the outer retina, S-opsin was increased. BMP7
36 expression in the mutant retina was strongly decreased at birth, but increased
37 expression from cells other than NC was observed in the adult retina. This was
38 associated with an increase in IBA1, suggestive that loss of NC-derived BMP7
39 predisposes to development of gliosis-like changes in the adult retina.

40 Overall, our data reveal an important contribution of NC-derived BMP7 for the
41 development and function of the inner and outer retina.

42

43 **1. Introduction**

44 Derivatives of neural crest cells (NCCs) contribute to the formation of various
45 structures in the adult eye, most notably the corneal endothelium and stroma of cornea,
46 iris, and ciliary body (Gage et al., 2005; Whikehart, 2010; Williams and Bohnsack,
47 2015). Eye defects resulting from improper migration or differentiation of NCCs have
48 mostly been associated with anterior segment dysgenesis syndromes and congenital
49 glaucoma (Langenberg et al., 2008; Williams and Bohnsack, 2015). NCCs also
50 contribute to the posterior segment, in particular pericytes of the transient choroidal
51 hyaloid vasculature (Etchevers et al., 2001). Some NCC-derived cells persist in the
52 adult retina as pericytes and vascular smooth muscle cells in the ganglion cell layer
53 (GCL), the inner plexiform layer (IPL), and inner nuclear layer (INL) during postnatal
54 stages (Trost et al., 2013). Furthermore Liu and colleagues have suggested that some
55 lineage traced NCCs express markers for Müller glia, raising the possibility that a subset
56 of Müller glia are NC-derived (Liu et al., 2014). Currently, no specific function has been
57 described for these NC-derived retinal cells.

58 The adult retina is derived from neuroepithelium and pigmented epithelial cells
59 that sequentially differentiate into 7 different layers: GCL, IPL, INL, outer plexiform layer
60 (OPL), outer nuclear layer (ONL), inner segments/outer segments (IS/OS) and retinal
61 pigment epithelium (RPE). The ganglion, horizontal, amacrine, and cone photoreceptor
62 cells are the first to develop and mature, followed by rod photoreceptors, bipolar cells
63 and Müller glia (Bassett and Wallace, 2012; Jeon et al., 1998), the major glial cell type
64 of the retina. The retina converts light information into electrical signals and transmits
65 the information to the visual processing centers in the brain via the process of

66 phototransduction. Light travels through the eye to the photoreceptors where a
67 signalling cascade hyperpolarizes the photoreceptors. The photoreceptors synapse
68 onto rod and cone bipolar cells, which then synapse onto the ganglion cells from where
69 the electrical information travels to the brain (Masland, 2012). Within this process,
70 Müller glia have several functions. Regularly spaced and spanning the entire thickness
71 of the neuroretina, they are involved in regulating potassium ion flux, serve as structural
72 support for neurons, and modulate neuronal and neurovascular coupling (Reichenbach
73 and Bringmann, 2013). In contrast to mammals, zebrafish Müller glia maintain the life-
74 long potential to regenerate all major retinal cell types (Hoang et al., 2020; Martins et
75 al., 2020).

76 In the brain, the NC-derived meninges provide critical trophic support during
77 embryonic neural development by regulating the neurogenic properties of radial glia
78 cells (Segklia et al., 2012; Siegenthaler and Pleasure, 2011), the life-long neurogenesis
79 in the dentate gyrus, and dentate neural stem cells (Choe et al., 2013). This process is
80 in part mediated by BMP7, an important signalling molecule needed to maintain normal
81 PAX6 expression, including after lens placode induction (Segklia et al., 2012; Wawersik
82 et al., 1999). BMP signaling also controls Müller glia differentiation, and the exposure of
83 retinal astrocytes and Müller glia to BMP7 results in reactive gliosis (Dharmarajan et al.,
84 2014; Wawersik et al., 1999). *BMP7* mutations in humans are associated with various
85 ocular abnormalities ranging from anophthalmia to microphthalmia (Wyatt et al., 2010).
86 Complete loss of BMP7 in mice results in anophthalmia (Zouvelou et al., 2009),
87 highlighting its significance for eye development. Despite this, little is known about the
88 requirement of BMP7 for the adult retina. No genetic studies using tissue-specific

89 deletion of BMP7 in the retina have been performed. Given that NC-derived BMP7 is
90 important for neural development and stem cell maintenance, and a subset of Müller
91 glia might be NC -derived, we asked whether BMP7 is expressed in the adult retina and
92 whether NC-specific BMP7 deletion would affect retina development and function.

93 In this study, we demonstrate that BMP7 remains expressed in various
94 compartments of the adult eye, including the retina. Loss of BMP7 in NCC ($Bmp7^{ncko}$)
95 results in loss of NCC in the GCL and INL, discrete cell organization defects in the outer
96 retina, and abnormalities to neuronal circuitry in the inner retina. NC-specific BMP7 loss
97 leads to increased expression of BMP7 by non-NC-derived expression in the adult
98 retina. Correlating cellular and molecular changes in the mutant retina to changes in
99 light perception and signalling using electroretinogram (ERG), we identified a
100 requirement of NC-derived BMP7 for normal vision. To our knowledge, this is the first
101 description of how an NC-derived growth factor ensures normal function of the adult
102 retina. Our data not only demonstrate how subtle changes to cellular and molecular
103 properties translate to visual defects in the adult eye, but also offer possible new
104 avenues for exploiting the plasticity of the retina to potentially correct retinal dysfunction.
105

106 **2. Methods**

107 **2.1. Ethics Statement**

108 All procedures were conducted in accordance with the Canadian Council on Animal
109 Care guidelines and approved by the Animal Care and Use Committee of the University
110 of Alberta (protocol: AUP1149).

111

112 **2.2. Animals**

113 Bmp7LacZ reporter mice (Godin et al., 1998), BMP7 neural crest knock-out mice
114 ($Bmp7^{fl/fl;Wnt1^{cre}}$, subsequently referred to as $Bmp7^{ncko}$ or mutant mice) were generated
115 as previously described (Malik et al., 2020; Zouvelou et al., 2009). Mouse lines were
116 kept on a C57BL/6J background. $Bmp7^{fl/fl}$ mice served as controls ($Bmp7^{ctrl}$), except for
117 lineage tracing, where $Bmp7^{wt;Wnt1^{cre}}$ mice were used. Lineage tracing was done using
118 mT/G mice ($Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$) (Muzumdar et al., 2007). Due to
119 the exploratory nature of this study, *a priori* power was not calculated, but post-hoc
120 power and effect sizes are provided. Male and female mice were used for all
121 experiments. For histological/immunofluorescence analysis, a minimum of three
122 biological replicates were analyzed.

123

124 **2.3. LacZ Staining**

125 LacZ staining of $Bmp7^{Wt:LacZ}$ enucleated mouse eyes were performed as previously
126 described (n=3/age) (Malik et al., 2020). LacZ stained eyes were fixed using Davidson's
127 fixative solution (Richmond, n.d.) as described below.

128

129 **2.4. Tissue Preparation and Histology**

130 Animals were euthanized, and enucleated eyes were fixed with Davidson's fixative
131 solution (Richmond, n.d.) for 12 hours, stored in 50% ethanol, and processed for
132 paraffin embedding and sectioning, as previously described (Baddam et al., 2020). Eyes
133 were sectioned in the sagittal orientation at a thickness of 7 μ m. Sections 70 to 140 μ m
134 ventral to the optic nerve were used for staining. Hematoxylin and eosin staining was
135 performed as previously described (Ellis, n.d.; Malik et al., 2020)

136

137 **2.5. Immunofluorescence**

138 Molecular markers were investigated in Bmp7^{ctrl} and Bmp7^{ncko} mice (n=3) and
139 lineage tracing experiments were conducted in Bmp7^{ctrl}, Bmp7^{ncko}, and Bmp7^{Wt:Wnt1cre}
140 mice (n \geq 3). Immunofluorescence and imaging were done as previously described using
141 paraffin sections (Malik et al., 2020). Sections were deparaffinized. Antigen retrieval
142 was performed using 10mM sodium citrate buffer in a microwave. Sections were
143 blocked with the appropriate goat or donkey serum in Tris-buffered saline (TBS).
144 Primary antibodies were incubated at 4°C overnight. Primary antibodies used were:
145 Calbindin (Calb; Santa Cruz, sc28285), Neurofilament Heavy (NF-H; Abcam,
146 ab187374), Glial Fibrillary Acidic Protein (Gfap; Abcam, ab4674), Pax6 (prb-278P),
147 Rhodopsin (Rho; Abcam, ab98887), Recoverin (Rcvrn; Abcam, ab5585), Blue Opsin (S-
148 op; short-wavelength; Millipore Sigma, ab5407), Red & Green Opsin (R&G-op; medium
149 wavelength; Millipore Sigma, ab5405), Tomato Lectin (Invitrogen L32470 DyLight 488),

150 Bone Morphogenetic Protein 7 (Bmp7; Abcam, ab84684) and Green Fluorescent
151 Protein (Gfp; Abcam, ab6556, provided by Luc Berthiaume, Department of Cell Biology,
152 University of Alberta). Secondary antibodies were: donkey anti-rabbit Alexa Flour (AF)-
153 647 (6440-31), goat anti-mouse IgG1AF-647 (A21240), goat anti-mouse IgG2a AF-647
154 (A21241), goat anti-mouse IgG3 AF-594 (A21155), and goat anti-chicken AF-594
155 (ab150172), donkey anti-chicken AF-488 (AB_2340375). Slides were mounted using
156 2.5% DABCO Mowiol and stored in the dark to avoid bleaching, at room temperature.
157 All images were captured on an Olympus IX73 microscope at 20x magnification, except
158 for S-op and R&G-op which was also imaged at 10x. These images were compiled and
159 overlaid using FireAlpaca (v. 2.2.10). Photoreceptors were counted from three
160 independent biological replicates. Whole retinal sections were used to count
161 photoreceptors positive for S-op (Blue cones) and R&G-op (Red and Green cones). A
162 single rater counted the photoreceptors and was blinded to the genotype of the mouse.
163 Image intensities were normalized to DAPI staining, and true staining was confirmed
164 through comparison to antibody negative controls and auto-fluorescent controls. All
165 slides were incubated in antibodies for the same length of time, and were imaged at the
166 same parameters (i.e., intensity, gain, exposure).

167

168 **2.6. Optical Coherence Tomography (OCT)**

169 One-month-old (P30) Bmp7^{ctrl}, Bmp7^{ncko} (n=4/genotype) were anesthetized by
170 intraperitoneal injection of 150mg/kg ketamine /10mg/kg xylazine; phenylephrine and
171 tropicamide were applied to dilate the pupil and prevent dryness. Mice were placed into

172 a stereotactic rotational cassette and the retina was scanned using the Bioptigen Envisu
173 spectral domain ophthalmic imaging OCT system. Data analysis was done using
174 InVivoVue Clinic imaging software. To measure retinal thickness, calipers were drawn
175 across all retinal layers (RNFL: retina nerve fiber layer; GCL: ganglion cell layer; IPL:
176 inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer
177 nuclear layer; IS/OS: inner segments/outer segments; RPE: retina pigmented
178 epithelium), for both the superior and inferior retina. Each mouse (2 eyes per mouse)
179 was evaluated by two researchers with high intra- (kappa: 0.94) and inter-rater reliability
180 (ICC: 0.83) blinded to mouse genotype at the time of assessment. Due to poor image
181 quality of the inferior retina, one $Bmp7^{ncko}$ mouse was excluded from all analysis for the
182 inferior retina.

183

184 **2.7. Electroretinogram (ERG)**

185 Mice were anesthetized by intra-peritoneal injection of 150mg/kg ketamine
186 /10mg/kg xylazine. Scotopic conditions were tested first, followed by photopic conditions
187 using 10 μ s light flashes (0.3-300Hz bandpass without notch filtering). Scotopic flashes
188 ranged from -5.22 to 2.86 log cd s/m², and photopic flashes ranged from -1.6 to 2.9 log
189 cd s/m² in 30 cd/m² background illumination. Body temperature of the mice was
190 maintained at 38°C. Dark (scotopic) and light (photopic)-adapted electroretinography
191 (ERG) was performed as previously described (Cheng et al., 2020) on a total of
192 nineteen P30 mice per group (N=38) using the Espion E 2 system (Diagnosys, LLC,
193 Littleton, MA). For scotopic conditions, mice were dark-adapted for 2 hours prior to

194 measurement and handled under dim red light. Researchers were blinded for mouse
195 genotype during measurement and analysis.

196

197 **2.8. Mining of scRNA Seq data**

198 The scRNA seq expression matrix of mouse retinal progenitor cells across 10 time-
199 points of retinal development (GEO Accession ID GSE118614, along with its metadata)
200 (Clark et al., 2019) was processed using Seurat to normalize data allowing comparison
201 between cells using Log Normalization, detection of genes with variable expression
202 patterns, and clustering and identification of cluster-specific marker genes to annotate
203 the cells with cell types. Results were visualized using non-linear embedding methods
204 including tSNE and UMAP, and a sparse matrix R data file (.rds) file was generated.
205 Scesay was used to convert the file format from a Seurat's .rds object to an annData's
206 .h5ad object, which was then loaded into an interactive single cell transcriptome data
207 explorer, cellxgene. The processed data was then loaded into the visualizer using the
208 command: cellxgene launch retina_ids_11_final.h5ad --open. The link to the processed
209 data file is available for access using <https://github.com/PriyankaMaripuri/Retina>.

210

211 **2.9. Statistics**

212 *A priori* power was not calculated as this was an exploratory analysis. Post-hoc
213 power analyses are provided for all significant results. The Shapiro-Wilk test for
214 normality was used to determine normality. Photoreceptor counts, OCT retinal layer

215 lengths, and ERG comparisons were assessed using unpaired t-tests. For ERG
216 analyses, intensities below the detection limit (0 amplitude response) were omitted from
217 analyses. All data are presented as mean \pm SD.

218

219 **3. Results**

220 **3.1. BMP7 expression is dynamic and overlaps with NCCs in the post-** 221 **natal eye**

222 To understand when and where BMP7 is expressed in the post-natal eye, its
223 expression was mapped using Bmp7LacZ reporter mice. At birth (Post-natal day 0, P0),
224 no expression was observed in the cornea (Figure 1A), but clear expression was seen
225 in the ciliary body (Figure 1B) and the RPE of the neural retina (Figure 1C).

226 At P14, when eyes open and rapid eye growth is observed in mice (Shen and
227 Colonnese, 2016; Tkatchenko et al., 2010), both the corneal epithelium and
228 endothelium expressed BMP7 (Figure 1D). Expression in the growing ciliary body
229 persisted and appeared to extend anterior (towards to the iris) and posterior (towards
230 the developing GCL of the retina) (Figure 1E). Within the retina, BMP7 was strongest in
231 the IS/OS layer, but expression extending to the OPL and GCL was apparent (Figure
232 1F).

233 At P30, BMP7 expression in the corneal epithelium became more restricted into
234 clusters, while expression in the endothelium appeared comparatively weaker (Figure
235 1G). In the ciliary bodies, expression remained ubiquitous (Figure 1H), whereas in the

236 retina BMP7 expression was limited to the GCL (Figure 1I). High magnification images
237 of Bmp7 expression demonstrated in Figure S1.

238 To assess which of these BMP7 expression domains overlapped with neural
239 crest-derived cells and structures, lineage tracing was performed using P30
240 $Bmp7^{Wt;Wnt1cre}$ retinas (n=3 mice). NCC-derivatives could be observed in stroma and
241 endothelium of the cornea (Figure 1J), the pigmented cells of the iris and within the
242 ciliary bodies extending towards the retinal pigmented epithelium (Figure 1K). Within the
243 retina, lineage-traced cells were apparent in the GCL, IPL, INL and OPL (Figure 1L).
244 A negative control for NCC staining is demonstrated in Figure S2. The partial overlap
245 between lineage tracing and BMP7 expression prompted us to assess if NC-derived
246 BMP7 is important for postnatal eye development and function.

247

248 **3.2. $Bmp7^{ncko}$ mice display molecular abnormalities, but no structural** 249 **differences**

250 Gross morphological analysis revealed that eyes of $Bmp7^{ncko}$ mice were of
251 comparable size. No apparent alterations to the cornea were apparent, but we noted
252 that pupils were slightly smaller, although not significant, after pharmacological dilation
253 (Figure S3). To gauge if there were any obvious structural defects in the retina, we
254 characterized the retina using OCT and histology. Whereas no statistically significant
255 changes to the retinal layers was observed at either inferior ($p \geq 0.06$) or superior
256 ($p \geq 0.13$) positions (Figures 2A-D), histological assessment identified some mild
257 disorganization of neuronal cell bodies in the ONL suggesting disrupted photoreceptor

258 stacking when compared to controls (Figure 2E-F). A reduction or loss of NC-derived
259 cells was observed specifically in the GCL and INL of $Bmp7^{ncko}$ retinas compared to
260 $Bmp7^{Wt:Wnt1cre}$ mice, whereas NC-derived cells at the OPL appeared unaffected. Loss of
261 BMP7 did not affect cellularity of NC-derived components lining the RPE (Figure 2G-H).
262 We thus asked next if loss of BMP7 resulted in functional impairments associated with
263 inner and outer retina.

264

265 **3.3. $Bmp7^{ncko}$ mice exhibit inner retina deficits**

266 ERG assessments performed on P30 $Bmp7^{ctrl}$ (black) and $Bmp7^{ncko}$ (red) mice
267 (n=19/genotype) revealed significant reductions in the a-wave (indicator of outer retina
268 function (Creel, 1995)) under scotopic conditions (Figure 3A; p=0.04), but not under
269 photopic conditions (Figure 3B; p=0.22, Cohen's d= 4.79). In contrast, the amplitudes of
270 the b-wave (an indicator of inner retina function (Creel, 1995)) under both scotopic
271 (Figure 3C; p=0.0009, Cohen's d=8.8 with 100% power) and photopic conditions (Figure
272 3D; p=0.008, Cohen's d= 8.49 with 100% power) were reduced. The reduced ratio of
273 scotopic b-wave/a-wave pointed towards a more pronounced inner retina defect (Figure
274 3E; p=0.002, d=1.72 with 99% power). The flicker response, which identifies the status
275 of cone photoreceptor function (Verma and Pianta, 2009), also showed a significant
276 reduction (Figure 3F; p=0.05, d= 7.5 with 100% power) reflecting decreased function of
277 cone photoreceptors. The implicit time, a measure of the time it takes to generate a- or
278 b- wave signals (Creel, 1995), was not significantly different for the a-wave under both
279 scotopic and photopic conditions (Figure 3G-H; p<0.70), suggestive of normal

280 phototransduction. The implicit time for the scotopic b-wave was significantly shorter
281 (Figure 3I; $p=0.01$, $d=1.52$ with 99% power), while the photopic b-wave implicit time
282 showed no significant difference (Figure 3J; $p=0.91$). The selectivity and severity of the
283 various visual deficits observed by ERG were surprising and could not easily be
284 explained by a mildly disorganized ONL. We thus sought to further characterize the
285 molecular and cellular composition of the inner and outer retina using selected antigen
286 markers by immunofluorescence.

287

288 **3.4. Abnormal neuronal circuitry in the inner retina of P30 *Bmp7*^{ncKO}** 289 **mice**

290 As visual deficits in mutant mice were primarily associated with inner retina
291 function, we assessed first the neuronal organization of the retina. DAPI stain is shown
292 for reference (Fig. 4A-B). Calbindin (CALB), a calcium-binding protein broadly
293 expressed in bipolar, amacrine, ganglion, and cone photoreceptor cells (Gu et al., 2016;
294 Morona et al., 2008) was reduced in the GCL and INL of mutant mice (Figure 4C, D).
295 Heavy chain neurofilament (NF-H), which stains retinal ganglion cells to assess
296 neuronal damage (Kashiwagi et al., 2003), was increased in the GCL (Figure 4E-F).
297 Glial fibrillary acidic protein (GFAP), which stains glial cells and astrocytes (Sarthy et al.,
298 1991), revealed potential differences to cell composition in IPL and OPL in the mutant
299 (Figure 4G-H, G'-H'). Ionized calcium binding adaptor molecule 1 (IBA1), a microglia
300 maker (Tassoni et al., 2015), was increased in GCL, INL and ONL in *Bmp7*^{ncKO} mice.
301 Paired box protein 6 (PAX6), which is largely confined to retinal ganglion cells and

302 amacrine cells at this age (Figure 4I-J) (Li et al., 2007; Riesenberg et al., 2009), was
303 markedly reduced in the GCL and INL (Figure 4I-J). The disruption of normal CALB, NF-
304 H and PAX6 expression in the GCL and INL, as well as the differences in glial cell
305 organization, suggest atypical neuronal circuitry of the inner retina.

306

307 **3.5. Impaired organization of cone photoreceptors in the outer retina** 308 **of Bmp7^{ncko} mice**

309 Next, we assessed potential molecular changes that would correlate with the a-
310 wave findings. DAPI stain is shown for reference (Fig. 5A-B). Rhodopsin (RHO) (Figure
311 5C-D) and Recoverin (RCVRN) (Figure 5E-F) are predominantly expressed in rod
312 photoreceptors that govern vision in dim light (scotopic conditions). RHO was reduced
313 in the IS/OS layer, while RCVRN was reduced in both the ONL and IS/OS layers,
314 indicating an effect of NC-derived BMP7 on photoreceptors. Wavelength-specific cone
315 photoreceptors that govern colour vision under bright light (photopic conditions) also
316 showed changes. Blue opsin (S-OP) confined to the ventral aspect of the retina (Nadal-
317 Nicolás et al., 2020; Ortín-Martínez et al., 2014) showed an increase in the IS/OS layer
318 of the Bmp7^{ncko} retina (Figure 5G, H). Red and Green opsin (R&G-OP) showed no
319 apparent numerical changes (Figure 5I, J). Quantification confirmed a significant
320 increase of S-OP in Bmp7^{ncko} retina (Figure 5K; $p=0.01$, $d=3.71$ with 92% power), with
321 no changes to the number of R&G-opsin expressing cones (Figure 5L; $p=0.14$). The
322 cumulative deficits to neuronal circuitry in the inner retina and rod/cone photoreceptor
323 abnormalities in the outer retina indicate that NCC-derived BMP7 exerts effects on

324 multiple cell types in the adult retina. However, it is still unclear which retinal cells are
325 NC-derived and when NC-derived BMP7 is required.

326

327 **3.6. Persistent neural crest cells associate with cells in the INL and** 328 **ONL in addition to the vasculature**

329 Earlier studies established that some NC-derived cells persist in the inner nuclear
330 and ganglionic cell layers of the retina as vasculature-associated pericytes to at least 6-
331 weeks of age (Trost et al., 2013). To test if all NCCs associate with vasculature
332 pericytes or whether some cells contribute to other cell types, we colocalized NCCs
333 (GFP lineage tracing) with vasculature (Tomato Lectin (TL)), neurons (NF-H) and glial
334 cells (GFAP). Colocalization with GFP and TL in P0, P14 and P30 $Bmp7^{Wt:Wnt1cre}$ mice
335 revealed that NCCs were frequently associated with vasculature (Figure 6A) but not all
336 vasculature associated with NCCs. We also observed cells, predominantly at P0 and
337 P14 that did not associate with vasculature, as evident by absent TL staining (Figure 6A
338 white arrows). We also occasionally observed a striped pattern of GFP positive cells in
339 1 of 3 $Bmp7^{Wt:wnt1cre}$ mice at all three ages investigated. Higher magnification images for
340 Figure 6A as shown in Figure S4.

341

342 **3.7. NCC-specific deletion of BMP7 increases BMP7 expression in the** 343 **mature retina**

344 Findings from *Bmp7*LacZ reporter mice (Figure 1) showed much broader BMP7
345 expression than could be attributed to NCCs. To test whether NC-specific BMP7
346 deletion results in changes of BMP7 expression in the retina, we probed for BMP7
347 expression in P0, P14, and P30 (n=2 at P30, n=1 at P0 and P14) *Bmp7*^{ctrl} and *Bmp7*^{ncko}
348 retinas (Figure 6B). At P0, BMP7 was broadly expressed except at the interface
349 between the GCL and NBL (neuroblastic layer). At P14 and P30, BMP7 expression
350 appeared weaker and was restricted to the GCL, OPL and ONL. In P0 *Bmp7*^{ncko}, BMP7
351 expression was consistently reduced (n=4) but expression was variable ranging from
352 near absent to weak expression (compare Figure 6A, C). At P14, expression was
353 mostly comparable to control mice although increased expression in the GCL was
354 noted. BMP7 expression at P30 was clearly increased in *Bmp7*^{ncko} mice, particularly in
355 the GCL, OPL and ONL. This dynamic change in expression was unexpected. Whereas
356 in the *Bmp7*^{ctrl} retina BMP7 expression was reduced with the maturation of retina,
357 expression in the *Bmp7*^{ncko} retina increased with maturation. Since BMP7 was strongly
358 reduced in the *Bmp7*^{ncko} retina at P0, we investigated whether its loss affected the
359 development of neurons and glial cells (Figure 6C). Much of the BMP7 appeared to
360 associate with GFAP-positive glial cells, whereas no comparable colocalization was
361 observed with NF-H (n=3 mice). An inverse relationship of BMP7 with NF-H expression
362 was observed, whereas a direct relationship was found for GFAP when comparing
363 *Bmp7*^{ctrl} and *Bmp7*^{ncko} retinas. This non-NCC-specific expression of BMP7 prompted us
364 to query which retina cell types might be its source in the developing and adult retina.

365

366 **3.8. BMP7 expression throughout retina development occurs in cells**
367 **largely distinct from factors affected by its loss.**

368 Cell types expressing BMP7 and their relative location in the eye are summarized
369 in Figure 7, top panel. Meta-analysis of scRNA datasets for the retina at various
370 developmental stages (Clark et al., 2019) revealed the extent and cellular identity of
371 BMP7-expressing cells. BMP7 (red) was observed predominantly in early and late
372 retinal progenitor cells at embryonic and early post-natal stages. After birth, BMP7 was
373 mainly localized to amacrine cells and some bipolar cells with minimal expression in
374 retinal ganglion cells, cone and rod photoreceptors. Co-localization with markers
375 affected by loss of BMP7 (CALB, NF-H, PAX6, RHO, S-OP and RCVRN) during
376 embryonic retina development showed that expression of BMP7 overlapped with PAX6,
377 and to some degree with CALB and NF-H. Co-expression with rod/cone markers was
378 minimal (Figure 7, bottom panel).

379 Overall, our data establish that BMP7 is an important trophic factor for retinal
380 maturation and that NC-derived cells in the retina coordinate the development of
381 different retinal cellular components.

382

383 **4. Discussion**

384 BMP7 has long been known as a critical factor for early eye development (Dudley
385 and Robertson, 1997) In this study, we establish that BMP7 is expressed at postnatal

386 stages of eye development and in the adult eye, some of which overlap with NC-derived
387 structures. We show the functional importance of NC-derived BMP7 for the normal
388 organization and function of the adult retina. This is to our knowledge the first report
389 using a genetic approach to specifically address the role of BMP7 in a subset of cells in
390 the adult eye. It is also the first study to demonstrate a requirement for an NC-derived
391 growth factor for normal maturation and function of the retina.

392 BMP7 is expressed in the retina at various developmental stages based on the
393 meta-analysis of scRNA datasets (Clark et al., 2019). BMP7 expression was observed
394 in early and late retinal progenitor cells at embryonic and early post-natal stages. LacZ
395 reporting of BMP7 supports this wider expression of BMP7. Although much of this
396 expression is not confined to NC, in the postnatal eye, BMP7 is expressed in NC-
397 derived structures of the cornea, the ciliary body and the iris. It should be noted that
398 LacZ reporting identifies cells expressing the mRNA of the gene of interest while
399 antibody staining demonstrates protein localization. Similar to what has been shown for
400 the developing cortex (Segkilia et al., 2012), the actual localization of BMP7 can be quite
401 different from source of expression. BMP7 was recently shown to modulate corneal
402 stroma and epithelial cell functions (Kowtharapu et al., 2018), we did not note any
403 obvious changes to the cornea in *Bmp7^{ncko}* mice. BMP signaling is required for ciliary
404 body development and both BMP4 and BMP7 have been implied in this process (Zhao
405 et al., 2002) BMP4 loss-of-function studies failed to establish a direct role on ciliary body
406 development (Rausch et al., 2018). In *Bmp7^{ncko}* mice, pupils appeared smaller, but the
407 difference was not significant following their pharmacological dilation.

408 Based on persistence of NC-derived cells in the retina (Trost et al., 2016; Trost et
409 al., 2016), their implication in ocular repair (Liu et al., 2014), and the importance of NC-
410 derived BMP7 for neural stem cells and neurogenesis (Choe et al., 2013; Segklia et al.,
411 2012), we focused our functional analysis on the retina. Changes to the anterior
412 segment of the eye can affect functional analysis of the posterior segment, in particular
413 ERG readings (Gagné et al., 2010; Johnson et al., 2019; Miura et al., 2016). Overall, the
414 type and degree of differences observed in *Bmp7*^{hcko} mice for ERG measurements (a/b-
415 wave, scotopic/photopic, flicker/implicit time) strongly suggest that they are not solely
416 the consequence of changes in the anterior segment (Gagné et al., 2010; Johnson et
417 al., 2019; Miura et al., 2016), but the result of specific changes to the inner and outer
418 retina itself.

419 The reduced a-wave amplitude under scotopic conditions along with the
420 reduction in rhodopsin and recoverin point towards impaired rod photoreceptor
421 hyperpolarization (Figure 5) (Robson et al., 2003). Hyperpolarization can be affected if
422 cytoskeleton-mediated alignment of photoreceptors is altered (Eckmiller, 2004). BMP7
423 stabilizes microtubules via the activation of c-Jun N-terminal kinases in neuronal cells
424 (Podkowa et al., 2010); thus loss of BMP7 may similarly precipitate perturbed cone
425 photoreceptor arrangement. Increased numbers of S-opsin expressing cone
426 photoreceptors could be the consequence of a disorganized ONL. The a-wave
427 amplitude reductions point to both rod and cone phototransduction defects, which in
428 turn would contribute to b-wave amplitude reductions.

429 The b-wave amplitude and implicit time, reflecting inner retina function (Stockton and
430 Slaughter, 1989), were reduced under both scotopic and photopic conditions in

431 Bmp7^{ncko} mice. We observed a reduction in Calbindin, indicating changes to bipolar,
432 amacrine and horizontal cells, in agreement with our observed b-wave deficit.
433 Overexpression of the BMP antagonist Noggin1 results in a reduction of bipolar cells in
434 the INL (Messina et al., 2016). However, these changes appeared to be more
435 qualitative than quantitative.

436 The reduced b/a amplitude ratios point to additional dysfunctions in the inner
437 retina. Loss of NC-derived BMP7 led to increased retinal expression of BMP7 at P30.
438 BMP7 has been associated with reactive gliosis and neuro-inflammation (Dharmarajan
439 et al., 2017, 2014). Reactive gliosis is a universal reaction to neuronal injury (Buffo et
440 al., 2008), typically resulting in proliferative gliosis (Vázquez-Chona et al., 2011). In
441 contrast, hypertrophic gliosis is characterized by Müller cells getting larger while not
442 sending projections in the outer retina (de Melo et al., 2012). The increase in Iba1 in
443 P30 Bmp7^{ncko} retinas is in line with the development of a gliosis. The altered distribution
444 of GFAP and NF-H again points towards involvement of BMP7 in cytoskeletal
445 organization of glia and astrocytes. The reduction in PAX6 at P30 further indicates an
446 effect on amacrine and RGCs cells. This may be similar to what has been described in
447 the developing cortex, where loss of BMP7 affects radial glia progenitor cells (Segklia et
448 al., 2012). On the other hand, increased expression of GFAP is associated with de-
449 differentiation of Müller glia and regeneration of rod photoreceptors (Raymond et al.,
450 2006), and thus might reflect retina stress caused by impaired rod photoreceptor
451 function and disrupted neuronal circuitry (Lewis and Fisher, 2003; Raymond et al.,
452 2006). It could be that NC-derived BMP7 provides trophic support to various
453 neuroepithelial cells, similar to what has been described for the brain (Choe et al., 2013;

454 Segklia et al., 2012). The unexpected dynamic expression of BMP7 (variable reduction
455 at P0, comparable expression at P14 and particularly its increased expression in the
456 P30 mutant retina) underscores the importance of BMP7 for the adult retina.

457 Our data reveals an important role for NCC-derived BMP7 in the developing
458 retina. Lineage tracing was in line with previously described observations (Liu et al.,
459 2014; Trost et al., 2013). In particular, using both Sox10-Cre and Wnt1-Cre reporter
460 mice Liu et. al (Liu et al., 2014) suggested the existence of cells spanning the retina
461 reminiscent of glia cells, which were observed in some but not all of our experiments
462 (Figure 6A). There is controversy regarding the use of Wnt1-Cre to delete NCC, as
463 ectopic expression of WNT1 has been observed in the ventral midbrain of this mouse
464 (Lewis et al., 2013). A Wnt1-Cre2 mouse was developed to overcome this limitation
465 (Lewis et al., 2013); however, this mouse was not used in this study due to its still
466 insufficient characterization and emerging controversy on its faithfulness (Debbache et
467 al., 2018). While the Wnt1-cre mouse has been shown to identify mostly neural crest
468 cells, it cannot be formally excluded that Wnt1 may be expressed in a few non-neural
469 crest cells at some stage during retina development. To gauge if the ERG phenotype
470 observed in $Bmp7^{ncko}$ mice could be the direct result of ectopic Wnt1-Cre activation, we
471 tested heterozygote $Bmp7^{ncko}$ mice. Their ERG responses were comparable to $Bmp7^{ctrl}$
472 mice; hence, we have no reason to believe that phenotypic observations in the $Bmp7^{ncko}$
473 mice are a direct consequence of ectopic Wnt1 expression.

474 Loss of function mutations of BMP7 in humans are associated with ocular
475 defects, although no specific details on retina function were described (Wyatt et al.,
476 2010). At the same time, these patients showed various craniofacial defects overlapping

477 in spectrum with Stickler syndrome. $Bmp7^{ncko}$ mice also display craniofacial defects
478 reminiscent of Stickler syndrome (Baddam et al., 2021; Kouskoura et al., 2013).
479 Alterations to BMP signaling have been associated with Stickler syndrome (Nixon et al.,
480 2019), and the genes mutated in Stickler syndrome (Collagens II, IX, XI) are expressed
481 at reduced levels in BMP7-mutant embryos (data not shown). Stickler syndrome
482 patients often display reduced scotopic b-wave amplitudes (Kondo et al., 2020) similar
483 to $Bmp7^{ncko}$ mice. However, they demonstrate prolonged b-wave implicit times in
484 contrast to $Bmp7^{ncko}$ mice that show faster b-wave implicit times. Beyond those
485 associated with high myopia, the visual acuity deficits in Sticker Syndrome patients are
486 not understood; the cellular and molecular alterations underlying the deficits remain to
487 be characterized. Molecular findings from this study may provide clues to better
488 understand the functional deficits.

489 In summary, this study demonstrates for the first time that NCC, in part mediated
490 through secretion of growth factors such as BMP7, contributes to cell organization and
491 neuronal circuitry in the adult retina. The observed phenotype is likely the consequence
492 of early alterations to retina progenitors that manifest as visual function deficits post-
493 development, but also indicates a continued requirement for BMP7 in the adult retina.
494 Further studies are needed to unravel in more detail how neural crest contributes to
495 retina development and maturation.

496

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503

504 **6. References**

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743 **7. Figure Legends**

744 **Figure 1. Dynamic expression of BMP7 and NCC in the mouse eye.** Sagittal
745 sections of paraffin-embedded eyes counterstained with nuclear fast red from
746 Bmp7LacZ reporter mice demonstrate minimal expression of BMP7 (blue) in the cornea
747 **(A)**, ciliary body **(B)** and retina **(C)** at P0. At P14, BMP7 is expressed in the epithelium
748 and endothelium of the cornea **(D)**, the cells lining the lens and the ciliary body **(E)** and
749 the GCL, OPL and IS/OS of the retina **(F)**. Expression is sustained only in the cornea
750 epithelium **(G)**, ciliary body **(H)** and GCL of the retina **(I)** at P30. GFP
751 immunofluorescence staining(McCabe and Berthiaume, 1999) (white) for NCC using
752 Bmp7^{Wt;Wnt1cre} eyes revealed the presence of NCC in the stroma and endothelium of the
753 cornea **(J)**, ciliary body **(K)** and the GCL and INL of the retina **(L)** at P30. P0: postnatal
754 day 0; P14: postnatal day 14; P30: postnatal day 30; ep: epithelium; s: stroma; en:
755 endothelium; cb: ciliary body; nr: neural retina; gcl: ganglion cell layer; ipl: inner
756 plexiform layer; inl: inner nuclear layer; opl: outer plexiform layer; onl: outer nuclear
757 layer; is/os: inner segments/outer segments; rpe: retinal pigment epithelium; GFP:
758 green fluorescent protein.

759

760 **Figure 2. Bmp7^{ncko} mice demonstrate abnormal cell organization in ONL and loss**
761 **of NCC in the inner retina with no obvious changes to thickness of retina layers.**
762 **Retinal layer** thickness assessment using OCT imaging of P30 Bmp7^{ctrl} **(A)** and
763 Bmp7^{ncko} **(B)** mice (n=4/genotype) showed no differences to retinal layer thickness at
764 both superior **(C)** and inferior **(D)** locations. Mildly disorganized cell stacking (arrows) in
765 the ONL was observed in Hematoxylin and Eosin stained P30 Bmp7^{ncko} **(F)** retina

766 compared to Bmp7^{ctrl} (E). Loss of GFP-positive NCC (white) was observed in the GCL
767 and INL (arrows) of P30 Bmp7^{ncko} (H) mice in comparison to Bmp7^{Wt:Wnt1cre} (G) mice.
768 OCT: Optical coherence tomography; P30: postnatal day 30; rnl: retina nerve fiber
769 layer; gcl: ganglion cell layer; ipl: inner plexiform layer; inl: inner nuclear layer; opl: outer
770 plexiform layer; onl: outer nuclear layer; is/os: inner segments/outer segments; rpe:
771 retinal pigment epithelium; GFP: green fluorescent protein.

772

773 **Figure 3. Bmp7^{ncko} mice have a more pronounced inner retina functional deficit**
774 **than outer retina.** Electroretinogram of P30 mice (n=19/genotype) indicated a
775 significant reduction in a-wave (outer retina function) of Bmp7^{ncko} mice in scotopic (A)
776 but not photopic (B) conditions. Assessment of the b-wave (inner retina function) also
777 demonstrated a significant reduction under both scotopic (C) and photopic (D)
778 conditions. A reduction in the scotopic b/a ratio (E) and a delay in flicker response (F) of
779 Bmp7^{ncko} mice retina confirm the severity of the inner retina defect. The implicit time of
780 a-wave under both scotopic (G) and photopic (H) conditions demonstrated no significant
781 differences. However, a significant reduction in the implicit time of scotopic b-wave (I)
782 was observed, with no changes to implicit time of photopic b-wave (J). For all statistical
783 analyses, intensities that were too low for rats of both groups (0 amplitude response)
784 were omitted. Black: Bmp7^{ctrl}; Red: Bmp7^{ncko}. P30: postnatal day 30.

785

786 **Figure 4. Abnormal neuronal organization in the inner retina of Bmp7^{ncko} mice.**
787 Bmp7^{ctrl} (A, C, E, G, G', I, K) and Bmp7^{ncko} (B, D, F, H, H', J, L) retina were stained for
788 various neuronal proteins and DAPI for nuclei (A-B) using immunofluorescence.

789 Reduced expression of CALB **(C-D)** was observed in GCL and INL of the mutant retina.
790 Increased expression of NF-H **(E-F)** was observed in the GCL of $Bmp7^{ncko}$. A slight
791 increase of GFAP **(G-H)** expression was observed in the GCL. **(G'-H')** Higher
792 magnification images of the GFAP expression in the GCL. Expression of IBA1 **(I-J)** was
793 increased in GCL, INL and ONL layers of $Bmp7^{ncko}$. Expression of PAX6 **(K-L)** was also
794 reduced in the GCL and INL of the mutant mice. Immunostaining was performed on
795 paraffin-embedded P30 retina (n=3/genotype). CALB: calbindin; NF-H: neurofilament
796 heavy; GFAP: glial fibrillary acidic protein; IBA1: Ionized calcium binding adaptor
797 molecule 1; PAX6: paired box protein 6. gcl: ganglion cell layer; ipl: inner plexiform
798 layer; inl: inner nuclear layer; opl: outer plexiform layer; onl: outer nuclear layer; is/os:
799 inner segments/outer segments; rpe: retinal pigment epithelium. P30: postnatal day 30.

800

801 **Figure 5. Increase in blue-opsin expressing cone photoreceptors in the outer**
802 **retina of $Bmp7^{ncko}$ mice.** P30 $Bmp7^{ctrl}$ **(A, C, E, G, I)** and $Bmp7^{ncko}$ **(B, D, F, H, J)** retina
803 (n=3/genotype) were immune-stained for various proteins expressed in rod and cone
804 photoreceptor and DAPI for nuclei **(A-B)** using immunofluorescence. Staining for rod
805 photoreceptors using RHO **(C-D)** and RCVRN **(E-F)** demonstrated a reduction in IS/OS
806 layer and ONL of the $BMP7^{ncko}$ mice. Staining for cone photoreceptors using S-OP **(G-**
807 **H)** and R&G-OP **(I-J)** demonstrated an increase in blue cones and no significant
808 changes to red and green cones. Cone photoreceptor quantification indicated an
809 increase in blue-opsin expressing cones **(K)** with no difference in red and green opsin-
810 expressing cones **(L)** in $Bmp7^{ncko}$ mice. Immunostaining was performed on paraffin-
811 embedded P30 retina (n=3/genotype). RHO: rhodopsin; RCVRN: recoverin; S-OP: blue

812 opsin; R&G-OP: red and green opsin. gcl: ganglion cell layer; ipl: inner plexiform layer;
813 inl: inner nuclear layer; opl: outer plexiform layer; onl: outer nuclear layer; is/os: inner
814 segments/outer segments; rpe: retinal pigment epithelium. P30: postnatal day 30.

815

816 **Figure 6. Persistent neural crest cells associate with cells in the INL and ONL and**

817 **deletion of BMP7 increases BMP7 expression in the mature retina.** GFP and BMP7

818 were co-localized with tomato lectin (TL; **A, B**), NF-H, and GFAP (**C**) to test if all NCCs

819 associate with vasculature or other retina cell types. At P0 and P14, the majority of

820 GFP-positive cells colocalize with TL but few GFP-positive cells in the INL and ONL are

821 negative for TL (**A**, white arrows). A striped GFP staining pattern was observed in 1 of 3

822 $Bmp7^{Wt:wnt1cre}$ mice at P0 and P30, resembling findings presented in Liu et al., 2014.⁷

823 Similarly, BMP7 expression in P0, P14, and P30 and $Bmp7^{ncko}$ retina (**B**) revealed

824 expression being confined mostly to the GCL and OPL layers, where TL staining is also

825 visible. In $Bmp7^{ctrl}$ mice, BMP7 expression decreased as the retina matures, however in

826 $Bmp7^{ncko}$ mice, a dramatic loss of BMP7 was observed initially at P0 with a gradual

827 increase of expression evident at both P14 and P30 timepoints. Neuronal (NF-H) and

828 glial (GFAP) cell markers, were colocalized with BMP7 at P0 (**C**) where a significant

829 amount of BMP7 is associated with GFAP positive glial cells whereas no comparable

830 colocalization was observed with NF-H. NF-H: neurofilament heavy; GFAP: glial

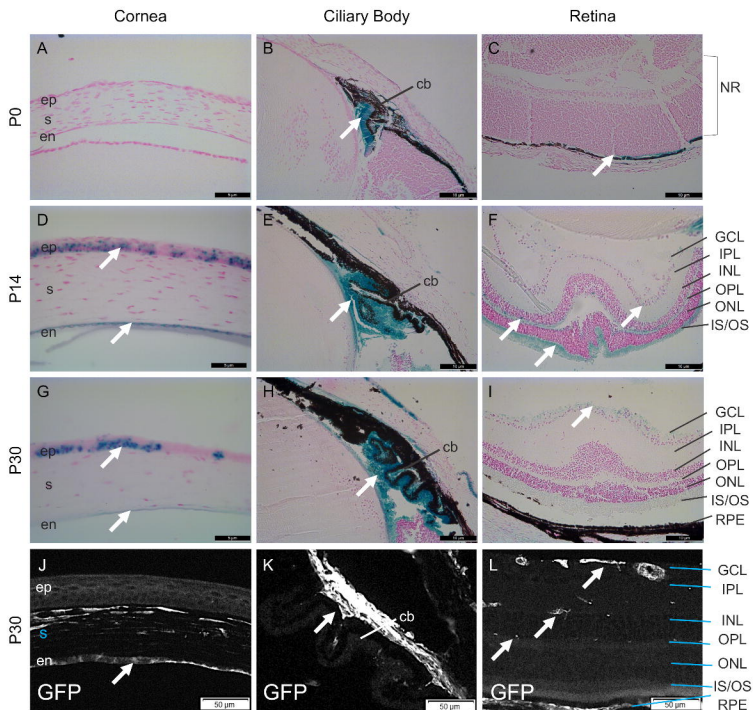
831 fibrillary acidic protein; GFP: green fluorescent protein; gcl: ganglion cell layer; inl: inner

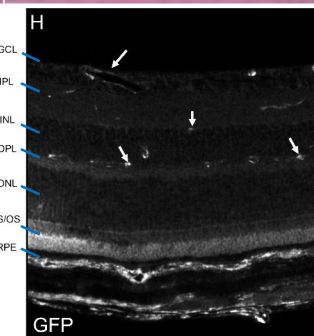
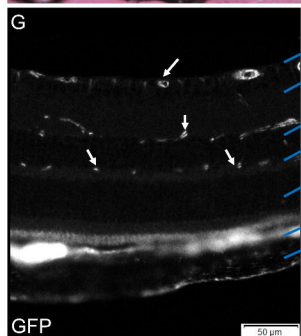
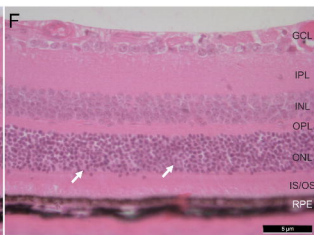
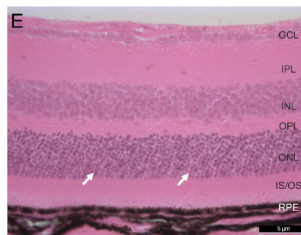
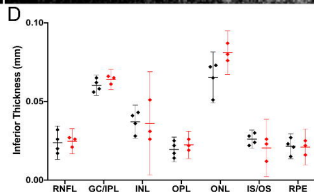
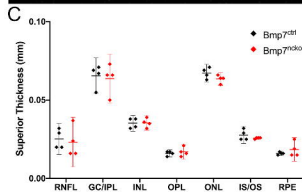
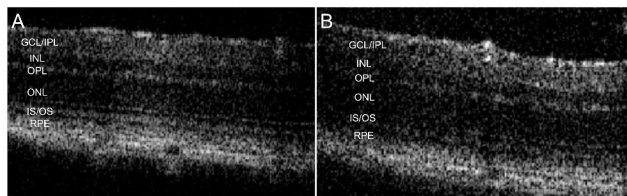
832 nuclear layer; opl: outer plexiform layer; onl: outer nuclear layer rpe: retinal pigment

833 epithelium. P0: postnatal day 0; P14: postnatal day 14; P30: postnatal day 30.

834

835 **Figure 7. Co-expression of BMP7 with various proteins expressed by neurons and**
836 **photoreceptors. (Top panel)** A summary of aberrant proteins expression observed in
837 P30 Bmp7^{ncko} mice retina. Increased expression of proteins in green and proteins with
838 decreased expression in red. BMP7 expression in blue. **(Bottom panel)** Data mining of
839 single cell RNA-Seq confirmed dynamic expression pattern of BMP7 during
840 development along with co-expression of proteins (CALB, NF-H, PAX6, RHO, S-OP,
841 RCVRN) assessed in this study. E: embryonic day; P: postnatal day. RHO: rhodopsin;
842 RCVRN: recoverin; S-OP: blue opsin; CALB: calbindin; NF-H: neurofilament heavy;
843 PAX6: paired box protein 6.
844





● Bmp7^{ctrl} ● Bmp7^{kncko}

