

1 Glycogen Synthase Kinase 3 regulates the genesis of the rare 2 displaced ganglion cell retinal subtype

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16 Running Title: **GSK3 negatively controls displaced ganglion cell genesis**

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22 **ABSTRACT**

23
24 Glycogen Synthase Kinase 3 (GSK) proteins (GSK3 α and GSK3 β) are key mediators
25 of signaling pathways, with crucial roles in coordinating fundamental biological
26 processes during neural development. Here we show that the complete loss of GSK3
27 signaling in mouse retinal progenitors leads to microphthalmia with broad
28 morphological defects. Both proliferation of retinal progenitors and neuronal
29 differentiation are impaired and result in enhanced cell death. A single wild-type allele
30 of either *Gsk3 α* or *Gsk3 β* is able to rescue these phenotypes. In this genetic context,
31 all cell types are present with a functional retina. However, we unexpectedly detect a
32 large number of cells in the inner nuclear layer expressing retinal ganglion cell (RGC)-
33 specific markers (called displaced RGCs, dRGCs) when at least one allele of *Gsk3 α* is
34 expressed. Excess dRGCs lead to increased number of axons projecting into the
35 ipsilateral medial terminal nucleus, an area of the brain belonging to the non-image-
36 forming visual circuit and poorly targeted by RGCs in wild-type retina. Transcriptome
37 analysis and optomotor response assay suggest that at least a subset of dRGCs in
38 *Gsk3* mutant mice are direction-selective RGCs. Our study thus uncovers a unique
39 role of GSK3 in controlling the genesis of dRGCs, a rare and poorly characterized
40 retinal cell type.

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42 Key words: Retinal development, Glycogen Synthase Kinase 3, Posttranslational
43 modifications, Displaced ganglion cells, Cell death, Proliferation

45 INTRODUCTION

46 Glycogen Synthase Kinase 3 alpha (GSK3 α) and beta (GSK3 β) are functionally
47 redundant serine/threonine kinases encoded by two different genes, sharing 95%
48 identity in their kinase domain (Doble et al., 2007). GSK3 exists at the crossroads of
49 multiple signaling pathways and acts as a key molecular switch to mediate their output
50 and guide distinct cellular processes (Cole, 2012; Doble and Woodgett, 2003;
51 Espinosa et al., 2003; Jin et al., 2009; Shimizu et al., 2008; Wang and Li, 2006). Among
52 the signaling pathways regulated by GSK3 kinases, Wnt canonical pathway is the most
53 well described, with GSK3 β inhibition triggering an increase in β -catenin protein levels
54 and its nuclear translocation to activate target gene expression (Doble and Woodgett,
55 2003).

56 GSK3 is a key regulator of neural stem/precursor cell proliferation in developing
57 as well as adult brain (Eom and Jope, 2009; Hur and Zhou, 2010; Kim et al., 2009;
58 Pachenari et al., 2017). Conditional deletion and gain of function experiments indicate
59 that GSK3 promotes neuronal differentiation (Hur and Zhou, 2010; Kim et al., 2009).
60 GSK3 exerts its effects through phosphorylation of key proteins involved in neural
61 development, including proneural factors such as Neurogenin 2 and NeuroD (Li et al.,
62 2012; Moore et al., 2002). In addition, GSK3 fine-tunes the balance between cell death
63 and survival, and its altered function is associated with neurodegenerative pathologies
64 including Alzheimer's disease, bipolar disorders, and Parkinson's disease (Golpich et
65 al., 2015; Jacobs et al., 2012; Kremer, 2011; Li et al., 2014; Maurer et al., 2014; Medina
66 et al., 2011).

67 GSK3 kinases are widely expressed in the developing retina (Pérezleón et al.,
68 2013). GSK3-dependent phosphorylation is shown to control the timing of proneural
69 factor activity and thereby regulate retinal cell fate determination. For instance,
70 inhibition of GSK3 signaling in the developing *Xenopus* retina leads to increase in
71 early-born cell types at the expense of late-born cells (Marcus et al., 1998; Moore et
72 al., 2002).

73 To elucidate GSK3 function in mammalian retina development, we generated
74 conditional loss-of-function alleles of *Gsk3 α* and *Gsk3 β* in retinal progenitor cells. We
75 show that complete loss of both GSK3 kinases severely impacts retinal morphology
76 with microphthalmia phenotype, which could be completely rescued with the
77 expression of just one *Gsk3 α* or *Gsk3 β* wild-type allele. We also noted the presence
78 of a large number of displaced retinal ganglion cells (dRGCs) in the inner nuclear layer
79 in the absence of either *Gsk3 α* or *Gsk3 β* . In normal conditions, this is a rare retinal cell
80 subtype, poorly characterized so far. Anterograde labeling of the axonal ganglion cell
81 projections into the brain of *Gsk3* mutant mice, allowed us to further support their
82 dRGCs identity. Our study thus identifies GSK3 as a possible determinant of dRGC
83 genesis. We also provide transcriptomic data and visual tests suggesting that at least
84 a subset of these supernumerary dRGCs in *Gsk3* mutant retinas are direction-selective
85 RGCs.

86

87 RESULTS

88 **Retinal progenitor-specific deletion of both *Gsk3 α* and *Gsk3 β* results in** 89 **microphthalmia**

90 We crossed the floxed *Gsk3 $\alpha^{ff}\beta^{ff}$* mice with *α -Cre* (*α Pax6-Cre*) line to generate
91 *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* mice in which *Gsk3* deletion occurs only in retinal progenitors as early
92 as E10.5 (Marquardt et al., 2001). We first validated our model by assessing the
93 efficacy of *Gsk3 α* and *Gsk3 β* deletion at E12.5 (Figure 1A). Immunohistochemistry
94 (IHC) using an antibody recognizing both GSK3 proteins showed ubiquitous
95 expression in control retinas (Figure 1A). Both *Gsk3* genes were efficiently deleted in
96 the peripheral retina of *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* mice, but their expression in the central retina
97 remained preserved consistent with the previously described *α -Cre* expression pattern
98 (Marquardt et al., 2001).

99 Hematoxylin and Eosin (H&E) staining revealed major morphological defects
100 with profound retinal disorganization, including the loss of radial arrangement as well
101 as folds and aggregates of retinal progenitor cells (RPCs), in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina
102 as early as E12.5 (Figure 1B). In addition, blood was detected inside the retinal
103 neuroblastic layer. Structure of the retina worsened rapidly during development
104 although the central part remained unperturbed, consistent with continued *Gsk3*
105 expression in this region. At and after E14.5, the retina was largely reduced whereas
106 the eye size itself was comparable to littermate controls (Figure 1B). A large quantity
107 of blood accumulated inside the eyeball at P2. Finally, growth of the eyeball was
108 severely reduced leading to microphthalmia in the adult (data not shown).

109 Because of a severe phenotype in the adult, we focused our histological
110 analyses to early retinal development. We first investigated subcellular localization of
111 β -catenin, an established GSK3 target that activates the canonical Wnt pathway (Doble
112 and Woodgett, 2003). In control retina, β -catenin expression was mostly cytoplasmic
113 (Figure 1C). Upon *Gsk3 α* and *Gsk3 β* deletion, β -catenin was still expressed in the
114 cytoplasm but translocated to the nucleus in some cells as early as E12.5.
115 *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina at E14.5 revealed a clearly defined boundary between the Cre-
116 negative region (GSK3-positive cells) at the center and the Cre-positive region (GSK3-
117 negative cells) at the periphery with nuclear translocation and large accumulation of β -
118 catenin in the nucleus (Figure 1C). Such an expression pattern is consistent with strong
119 activation of canonical Wnt pathway in the absence of both *Gsk3 α* and *Gsk3 β* . These
120 findings demonstrate that GSK3 regulates Wnt signaling in RPCs and is essential for
121 proper retinal development.

122

123 **Lack of both *Gsk3 α* and *Gsk3 β* in RPCs leads to cell cycle aberrations and retinal** 124 **progenitor gene deregulation**

125 We focused our analysis on early retinal development (E12.5 and E14.5) in
126 *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* mice to elucidate the cause of microphthalmia phenotype. To
127 investigate whether proliferation was altered in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* mouse retina, a single
128 dose of EdU (to label RPCs in the S-phase) was injected 16 hours prior to harvesting
129 embryos at E12.5 and E14.5. As predicted, EdU-positive cells increased significantly
130 from E12.5 to E14.5 in control retina (Figure 2A, B); yet, no change was detected in
131 *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina and reduction in RPCs in S-phase was evident at E14.5

132 compared to controls, suggesting proliferation defects. We further examined cell
133 proliferation by labeling late-G2/M-phase retinal progenitors using phospho-histone 3
134 (pH3) antibody. In control, strongly labeled pH3-positive cells were positioned in the
135 apical surface of the retina where mitosis occurs (M-phase cells). Cells with less
136 intense labeling, presumably late-G2 cells, were present in the outer part of the retinal
137 neuroblastic layer. In *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina, pH3-positive cells located at the
138 periphery were positioned distant from the apical side at both stages (Figure 2A).
139 Moreover, their number at the periphery was abnormal compared to controls, being
140 increased at E12.5 and decreased at E14.5 (Figure 2B). Double pH3- and EdU-positive
141 cells were increased in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retinas compared to controls, at both stages,
142 indicating aberrant cell cycle kinetics (Figure 2B). To investigate the consequence of
143 such cell proliferation defects on the pool of RPCs in the absence of GSK3, we
144 analyzed the expression of Hes1, a retinal progenitor marker (Wall et al., 2009). At
145 E12.5 and E14.5, anti-Hes1 antibody labeled all RPCs throughout control retina with
146 the exception of the basal side where differentiating ganglion cells are located (Figure
147 S1). In *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retinas, Hes1 expression was similar to controls at E12.5
148 indicating the maintenance of RPC pools (Figure S1A). However, by E14.5, Hes1
149 positive cells were sparser at the periphery where *Gsk3* expression was absent (Figure
150 S1B). In the same area lacking *Gsk3* expression, other progenitor markers, such as
151 Pax6 and Sox2, were similarly decreased in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina compared to
152 controls (data not shown). Thus, our results show that GSK3 kinases are required for
153 retinal progenitor proliferation and the maintenance of the pool of RPCs.

154

155 **Loss of *Gsk3 α* and *Gsk3 β* impairs retinal progenitor differentiation leading to** 156 **cell death**

157 To examine whether neuronal differentiation is impacted by the absence of
158 GSK3, we performed IHC using two neuronal markers, Doublecortin (Dcx) and Brn3a,
159 to label newly generated neurons and RGCs, respectively (Figure 3). At E12.5 and
160 E14.5, Dcx- and Brn3a-positive cells were localized in the inner part of the neuroblastic
161 layer of the control retina where retinal differentiation occurs first. In contrast, neuronal
162 differentiation was completely abolished at both stages in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina,
163 except in the most central part corresponding to the area that does not express Cre
164 recombinase. The absence of retinal differentiation and decreased expression of
165 proliferation markers prompted us to investigate whether the loss of *Gsk3* in RPCs
166 could trigger cell death. We indeed detected a significant increase in the number of
167 TUNEL-positive cells in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina at both embryonic stages (Figure S2).
168 Thus, microphthalmia observed in the absence of both *Gsk3 α* and *Gsk3 β* is likely due
169 to a succession of developmental defects ranging from impaired proliferation and
170 differentiation of RPCs to increased cell death.

171

172 **Multiple allelic combinations revealed functional redundancy of *Gsk3 α* and** 173 ***Gsk3 β* in retinal development**

174 Severe deleterious effect of the loss of both *Gsk3 α* and *Gsk3 β* in RPCs in early
175 development precludes the analysis of late retinal histogenesis. To circumvent this, we

176 generated animals with different combinations of *Gsk3* deletion (loss of only one *Gsk3*
177 gene: *Gsk3* $\alpha^{ff/\beta^{+/+};\alpha-Cre}$ or *Gsk3* $\alpha^{+/+\beta^{ff};\alpha-Cre}$, or $\frac{3}{4}$ deletion: *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ or
178 *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$). Immunoblot analysis using anti-GSK3 antibody (recognizing both
179 proteins) in 2-month-old animals with different combinations of *Gsk3* α and *Gsk3* β
180 floxed alleles demonstrated the efficacy of *Gsk3* α and *Gsk3* β deletion (Figure S3A).
181 IHC analysis using anti-GSK3 β showed ubiquitous expression of *Gsk3* β in adult
182 control retina and its complete loss in *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ retina (Figure S3B). At 2-
183 months, retinal histology revealed the correct laminated architecture with normal
184 photoreceptors and interneurons when even one allele of *Gsk3* α (Figure S3C, D) or
185 *Gsk3* β (data not shown) was present. Photopic and scotopic electroretinogram (ERG)
186 recordings, corresponding to cone and rod function respectively, did not show any
187 significant difference between *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ and control retina (Figure S3E, S3F).
188 These results were similar in mice carrying any combination of *Gsk3* deletion (data not
189 shown). We therefore conclude that a single allele of wild-type *Gsk3* α or *Gsk3* β is
190 sufficient to rescue obvious structural and functional defects in the complete absence
191 of GSK3 signaling.

192

193 **Loss of either *Gsk3* α or *Gsk3* β in RPCs leads to increased number of displaced** 194 **retinal ganglion cells**

195 Even though a single allele of either *Gsk3* α or *Gsk3* β permitted normal retinal
196 development (Figure S3), we observed a striking increase in the number of RGCs, as
197 indicated by Brn3a-positive cells, in the inner nuclear layer (INL) of *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$
198 retina compared to controls (Figure 4A). Brn3a-positive cells in the INL have been
199 described as displaced retinal ganglion cells (dRGCs), a rare cell type in the
200 mammalian retina (Galli-Resta and Ensini, 1996; Young, 1984). All Brn3a-positive cells
201 in the INL of *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ retina also expressed NF68 that labels cell bodies and
202 axons of RGCs (Figure 4A). Increased dRGCs were observed in retinas carrying any
203 combination of *Gsk3* deletions tested (*Gsk3* $\alpha^{ff/\beta^{+/+}$, *Gsk3* $\alpha^{+/+\beta^{ff}$, *Gsk3* α^{ff/β^{ff} or
204 *Gsk3* $\alpha^{ff/\beta^{ff/+}$), with the highest number detected in *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ mice compared to
205 controls (10-fold increase) (Figure 4B). Interestingly, increase in dRGCs number is not
206 associated with reduced number of RGCs in the GCL, referred to as orthotopic RGCs
207 (oRGCs) (Figure 4B). To validate that these Brn3a-positive cells in the INL were indeed
208 RGCs with axonal projections included in the optic nerve, we performed retrograde
209 labeling with Rhodamine-Dextran applied onto the optic nerve of *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$
210 mice. Subsequent 3D reconstructions on flat mount retinas revealed the presence of
211 numerous fluorescent cell bodies located in the INL compared to controls,
212 demonstrating that axons of dRGCs indeed reached the optic nerve (Figure 4C). Thus,
213 Brn3a-positive cells located in the INL of *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ retinas are indeed RGCs.

214 Due to their low number in control retina (around 2%), dRGCs have been poorly
215 characterized with very few markers identified, such as Brn3a (Nadal-Nicolás et al.,
216 2012; Nadal-Nicolás et al., 2014). Immunostaining on sections and flat mount retinas
217 with additional RGC marker antibodies revealed that dRGCs in *Gsk3* $\alpha^{ff/\beta^{ff};\alpha-Cre}$ retina
218 were also positive for Rbpms (Rodriguez et al., 2014) confirming their increased
219 number in the INL compared to controls (Figure 4D, E). Similar results were observed

220 with Islet1 labeling (Figure S4) (Bejarano-Escobar et al., 2015). Finally, Brn3a-positive
221 dRGCs did not express markers of other INL neurons such as Choline-
222 Acetyltransferase (CHAT, amacrine cells), Calretinin (amacrine cells), or Calbindin
223 (horizontal cells) further confirming their RGC identity (data not shown).

224 To test whether dRGCs in *Gsk3* mutant mice were produced during the same
225 developmental time window as oRGCs, we performed pulse chase experiments by
226 injecting EdU at E12.5, at the peak of RGC birth. Retinal sections from one-month-old
227 animals were then immunolabelled using anti-Brn3a antibody (Figure 5A). In control
228 and *Gsk3 $\alpha^{fl/+}\beta^{fl/fl};\alpha$ -Cre* retina, we identified 40-50% of RGCs that were Brn3a/EdU-
229 positive in all layers examined (GCL and INL), indicating that both dRGCs and oRGCs
230 were born around the same time (Figure 5B). We next examined whether dRGCs are
231 usually overproduced during normal retinal development and eliminated later on. In
232 this context, increased number of dRGCs in *Gsk3 $\alpha^{fl/+}\beta^{fl/fl};\alpha$ -Cre* retinas could result from
233 a defect in dRGC riddance occurring during the first two postnatal weeks, a period of
234 developmental cell death in the retina (Young, 1984). At P0, the number of Brn3a-
235 positive oRGCs was similar between littermate control and *Gsk3 $\alpha^{fl/+}\beta^{fl/fl};\alpha$ -Cre* retina
236 (Figure 5C and D). In contrast, the proportion of Brn3a-positive cells located in the
237 inner part of neuroblastic layer corresponding presumably to dRGCs was much lower
238 in control retinas ($6\pm 0.1\%$) compared to *Gsk3 $\alpha^{fl/+}\beta^{fl/fl};\alpha$ -Cre* retinas ($30\pm 1.4\%$). Thus,
239 dRGCs are not overproduced and eliminated postnatally in control retina. Our results
240 demonstrate that dRGCs are generated during early waves of retinogenesis in
241 *Gsk3 $\alpha^{fl/+}\beta^{fl/fl};\alpha$ -Cre* retina and strongly suggest that GSK3 kinases play a role in
242 restricting their number during normal retinal development.

243 **dRGCs produced in the absence of either *Gsk3 α* or *Gsk3 β* project to accessory** 244 **visual system circuitry**

245 Previous studies in birds and reptiles have reported that dRGCs could be
246 responsible for optokinetic nystagmus, as they mostly project to the accessory optic
247 nuclei (AOS) (Cook and Podugolnikova, 2001), which is critical for non-image forming
248 circuit and image stabilization (Simpson, 1984). To test whether dRGCs in *Gsk3*
249 mutants project into specific visual nuclei in the brain, including the AOS, we traced
250 the total pool of RGCs, including dRGCs, with Cholera Toxin beta subunit (CTB). Bi-
251 lateral injection of CTB, coupled to either an alexa-555 or -647 followed by 3D imaging,
252 allowed us to trace both ipsi- and contra-lateral projecting axons. We first confirmed
253 that CTB injections indeed marked the dRGCs based on flat mounts of retinas after
254 Brn3a immunolabelling (data not shown). To visualize the entire visual projection
255 network, we carried out whole-brain clearing using iDISCO+ followed by light-sheet
256 fluorescent imaging and 3D reconstruction (Figure 6A). Complete loss of *Gsk3 β*
257 displayed a specific increase in ipsilateral projecting RGCs specifically in one of the
258 three nuclei composing the AOS, the Medial Terminal Nucleus (MTN) (Simpson,
259 1984). This terminal nucleus is the main component of the AOS reacting best to either
260 upward or downward movement and mediates the optokinetic nystagmus critical for
261 image stabilization (Yonehara et al., 2009). Calculation of the signal intensity ratio
262 between the ipsilateral and contralateral MTN demonstrated a significant increase of
263 RGC projections into the ipsilateral MTN in retinas with *Gsk3 β* deletion (Figure 6B).

264 This result strongly suggests that excess dRGCs might participate to the non-image
265 forming circuit.

266 **Whole transcriptome analysis suggests that dRGCs in GSK3 mutant retinas are** 267 **direction-selective ganglion cells.**

268 We next performed transcriptome analysis using RNA-sequencing in order to
269 identify molecular changes in adult *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* retina and to better characterize
270 dRGCs. Retinas from *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$* mice were used as controls. Gene level analysis
271 revealed 111 differentially expressed genes (DEGs) using filtering criteria of Fold
272 Change (FC) = 1.5 with a False Discovery rate (FDR) cutoff of ≤ 0.05 and a minimum
273 mean expression value of one FPKM (fragments per kilobase of exon per million reads
274 mapped) in at least one of the two experimental groups (Figure 7A and Figure S5).
275 Pathway analysis of DEGs revealed several statistically significant overrepresented
276 pathways (Figure S6). Biological processes and molecular functions pathways
277 included 48 DEGs; of these, 33 genes were expressed in RGCs based on published
278 whole transcriptomic data from purified RGCs (for a total 69 RGC-expressed genes
279 among the 111 DEGs) (labeled with stars in Figure 7B) (Sajgo et al., 2017). Dominance
280 of RGC-expressed genes in our dataset is consistent with the high number of dRGCs
281 observed in *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* retina.

282 Among interesting candidates dysregulated in the biological processes and
283 molecular function pathways (Figure 7B and S6), we identified *Chrna2*, *Chrna5*,
284 *Chrna7*, and *Chrnab4*. With the exception of *Chrna2*, all of these are reported in purified
285 ganglion cell transcriptomic data (Sajgo et al., 2017). RT-qPCR analysis validated
286 upregulation of *Chrna5*, as well as the downregulation of *Chrnab4* (Figure 7C). For
287 *Chrna7*, we could only find a trend toward the increase although not significant. We
288 hypothesize that upregulation of *Chrna5*, and potentially that of *Chrna7*, results from
289 the increase in dRGCs in *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* retina. Another gene *Cartpt*, encoding
290 preprotein CART (Cocaine- And Amphetamine-Regulated Transcript Protein) that was
291 upregulated in *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* retina was validated by RT-qPCR (Figure 7C). *Cartpt*
292 is specifically expressed in direction-selective RGCs (DS-RGCs) (Kay et al., 2011;
293 Rouso et al., 2016), suggesting that dRGCs (or at least a subset) in *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre*
294 retina might be DS-RGCs. In support with this hypothesis, we found some dRGCs in
295 *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* and littermate control retina positive for the transcription factor *Tbr2*,
296 described as essential for RGC specification participating in non-image-forming visual
297 circuits (Figure 7D) (Sweeney et al., 2014, 2019). A small subset of dRGCs also
298 expressed *Foxp2*, a transcription factor involved in DS-RGC differentiation in mice
299 (Figure 7D) (Rouso et al., 2016; Sato et al., 2017). These two factors were expressed
300 in a mutually exclusive way in Rpbms-positive dRGCs, suggesting that dRGCs in
301 *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* might encompass several subtypes.

302

303 **Optomotor response is impaired in GSK3 mutant**

304 Given that DS-RGCs are reported to drive the optomotor response (OMR) by
305 projecting mainly into the contralateral AOS (Simpson, 1984; Yonehara et al., 2009),
306 we tested the OMR of *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* mice. The OMR indices ($T_{\text{correct}} /$
307 $T_{\text{incorrect}}$) were calculated from three trials at contrast 1 and 0.5 (Figure 7E). At

308 100% contrast, the OMR indices were significantly reduced in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* mice
309 compared to controls at 0.05, 0.15 and 0.25 cycles per degree (cpd). The maximum
310 OMR index was observed at 0.15 cpd in controls whereas it reached its maximum at
311 0.1 in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* mice. At 50% contrast, the OMR indices were also significantly
312 reduced in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* mice compared to controls but to a larger extent between
313 0.05 and 0.3 cpd. The maximum OMR index was observed at 0.15 cpd in both controls
314 and *Gsk3 α ^{f/+} β ^{ff}; α -Cre* mice. Altogether, these results demonstrate an impaired OMR
315 in *Gsk3 α ^{f/+} β ^{ff}; α -Cre* mice. These data, in support with our transcriptomic and axonal
316 projections analyses, suggest that at least a subset of dRGCs expressing only one
317 allele of *Gsk3 α* are DS-RGCs.
318

319 DISCUSSION

320 In this study, we report that complete loss of GSK3 in retinal progenitors leads
321 to microphthalmia in adult mice resulting from a successive chain of catastrophic
322 molecular events causing severe morphological defects with progressive death of the
323 pool of proliferative retinal progenitors and lack of neuronal differentiation. Such a
324 severe phenotype was not observed anymore when only one *Gsk3 α* or *Gsk3 β* allele
325 was expressed, confirming the functional redundancy of these two genes. Our results
326 suggest that the kinase GSK3 is the first discovered dRGCs determinant during retinal
327 histogenesis. We indeed found that mouse retinas with only one allele of *Gsk3* exhibit
328 an excessive number of dRGCs. The concomitant large increase of axonal projections
329 to the ipsilateral MTN, our RNA-Seq data and optomotor response tests, led us to
330 propose that these dRGCs are involved in the detection of image motion direction.

331 We reported that complete loss of GSK3 activity in retinal progenitors leads a
332 successive chain of catastrophic molecular events causing severe morphological
333 defects with progressive death of the pool of proliferative retinal progenitors and lack
334 of neuronal differentiation. A lack of cell differentiation was also described in the
335 developing brain of *Gsk3* mutant mice (Kim et al., 2009). Surprisingly however, we
336 demonstrate that the absence of GSK3 impairs the maintenance of the pool of retinal
337 progenitors and their survival, leading subsequently to microphthalmia in adults. Our
338 results are in striking contrast with the “big head phenotype” due to a large expansion
339 of the pool of neural progenitors by hyperproliferation and the absence of cell death at
340 early stages observed in *Gsk3 α ^{-/-};Gsk3 β ^{ff}; Nestin-Cre* embryos (Kim et al., 2009).
341 Retinal progenitors might be more prompt to cell death if they encounter proliferative
342 defects compared to brain neural progenitors. GSK3 kinases are established negative
343 regulators of the Wnt canonical pathway (Doble and Woodgett, 2003). As such, we
344 found that lack of GSK3 leads to increased amount of β -catenin nuclear translocation.
345 Interestingly, the effect of *Gsk3* loss of function in the retina mimics the effect of β -
346 catenin gain of function. Indeed, constitutive retinal activation of β -catenin does not
347 elicit an hyperproliferation of retinal cells, as one could have expected from Wnt
348 pathway activation but instead, it decreases cell proliferation and neural differentiation,
349 resulting in a small eye phenotype (Fu et al., 2006; Ouchi et al., 2011). Besides, β -
350 catenin has been recognized as a key regulator of cell adhesion (Ouchi et al., 2011).

351 The lamination defects observed in *Gsk3* knockout mice may thus also result from
352 altered cell adhesion due to β -catenin activation.

353 In pigmented wild-type mouse retina, dRGCs in the INL are a very rare and
354 poorly-described type of cells representing only 2% of RGCs (Balkema and Dräger,
355 1990; Doi et al., 1994; Dräger and Olsen, 1980; Nadal-Nicolás et al., 2014). It is
356 therefore striking that dRGC number increases up to 20% when a single copy of *Gsk3 α*
357 is present in retinal progenitors. To our knowledge, such a high number of dRGCs has
358 never been reported in a transgenic/mutant animal. A previous study hypothesized that
359 dRGCs are misplaced in the INL due to an ontogenic aberration rather than
360 representing an independent class of RGCs (Buhl and Dann, 1988; Doi et al., 1994).
361 Indeed, differential cell adhesion plays a key role in sorting and migration of retinal
362 cells in their appropriate layers, especially for RGCs. One can therefore hypothesize
363 that enhanced dRGCs in mice with a single copy of *Gsk3* is the consequence of
364 increased aberration events. This hypothesis could be supported by our RNA-Seq data
365 showing the upregulation of genes coding for collagen subunits (*Col18a1*, *Col4a3*,
366 *Col9a1*, *Col9a2*) and extracellular matrix proteins in *Gsk3 α ^{fl/+} β ^{fl/fl}; α -Cre retina, which
367 could favor migration defects. Noticeably, if it were the case, the increase in dRGCs
368 should be accompanied by a decrease in oRGCs. However, we found that the number
369 of oRGC in the GCL is unaltered, strongly suggesting that RGCs in the INL of mice
370 with a single copy of *Gsk3* represent a specific subtype of RGCs. In support with this,
371 topographic and quantitative analysis of RGCs in albinos and pigmented rats indicate
372 that dRGCs are not misplaced by ontogenic mistakes but indeed represent a specific
373 subpopulation of RGCs (Nadal-Nicolás et al., 2014). GSK3 β is involved in neural cell
374 fate decision by controlling the timing of the activity of bHLH transcription factors, such
375 as NeuroD or Neurog2 (Li et al., 2012; Moore et al., 2002). If dRGCs are not produced
376 following ontogenic aberrations but are instead determined by a proper genetic
377 program, it would be interesting to identify transcription factors involved and seek for
378 any regulation by GSK3 kinases.*

379 In reptiles, amphibians and birds, only dRGCs project into the MTN, whereas in
380 mammals only oRGCs have been reported as projecting into the MTN (Fite et al., 1981;
381 Krause et al., 2014). Our results obtained from anterograde labeling clearly
382 demonstrated a large increase in ipsilateral MTN projections in absence of *Gsk3 β* ,
383 whereas it was absent or very dim in control animals. Although this strongly suggests
384 that excess dRGCs in mutant mice are causing this phenotype, we cannot exclude the
385 possibility that mutant oRGCs also participate to these ipsilateral MTN projections.
386 However, contralateral projections did not seem to be affected. We can speculate that
387 the low number of ipsilateral MTN projections in the control condition reflects the low
388 number of dRGCs present in the WT retina and could therefore explain that such result
389 had not been described so far. Altogether, our results strongly suggest that dRGCs
390 may primarily project into the ipsilateral MTN. In mice, it has been shown by retrograde
391 labeling from the superior colliculus (SC), which receive large amount of RGC
392 projections, that dRGCs/oRGCs project to one or both SCs (Karten et al., 1977).
393 Although challenging, similar experiments, *i.e.* fluorescent dye injection into the
394 ipsilateral MTN, may allow to discriminate whether the increased signal in absence of

395 *Gsk3 β* originates only from dRGCs and whether these cells also project into this area
396 in WT retina. In regards to our results, it is still unclear whether GSK3 function is to
397 limit the number of dRGCs and actively regulates their correct projection to the
398 contralateral MTN or if GSK3 function is limited to the tight control of dRGC number,
399 which project thereafter to the ipsilateral MTN on a GSK3-independent manner.

400 Given the very low percentage of dRGCs in the control retina, their function is
401 poorly studied in mammals. In contrast, dRGC function, projections and topography
402 have been extensively investigated in bird and reptile retina (Mouritsen et al., 2004). In
403 birds, cryptochrome-expressing dRGCs are used as a magnetic compass for
404 orientation (Nießner et al., 2016). In European Robin birds, *Erithacus rubecula*, a low
405 number of dRGCs have been identified but specifically express Cryptochrome 1b only
406 during nocturnal migration period (Nießner et al., 2016). In rodents, retrograde labeling
407 from the optic nerve led to the identification of 16 classes of dRGCs based on their
408 ramification levels of their dendrites as well as the dendritic field size (Pang and Wu,
409 2011). Based on dRGC dendrite projections into the IPL, it has been proposed that
410 most dRGCs in WT retina are functionally more involved in retinal OFF light pathways
411 (Pang and Wu, 2011). Similar methods applied to *Gsk3 $\alpha^{fl/+}$ $\beta^{fl/fl}$; α -Cre* retina should shed
412 more light on dRGC function and establish whether all the different classes are present.

413 As part of the AOS, the MTN receives afferent signal from the eye and sends
414 efferent signal to motor neurons controlling the position of the eye. As such, optokinetic
415 reflex relies on direction specific retinal projections to the AOS. Neurons of the dorsal
416 terminal nucleus (DTN) codes for horizontal stimulus whereas neurons of the MTN
417 codes for vertical stimulus (Giolli et al., 2006; Yonehara et al., 2009). Therefore, the
418 direction of image motion relies on DS-RGCs in the retina. The alteration the OMR in
419 *Gsk3 $\alpha^{fl/+}$ $\beta^{fl/fl}$; α -Cre* mice, support the hypothesis that some of the supernumerary
420 dRGCs are indeed related to motion detection. Although the number of dRGCs was
421 drastically increased, the OMR was not increased but at the contrary reduced. Such
422 result might be caused by the higher number of projections to the ipsilateral side
423 instead of the contralateral one, leading to an alteration of the neuronal circuit
424 regulating the OMR (Simpson, 1984; Yonehara et al., 2009). We also identified in
425 *Gsk3 $\alpha^{fl/+}$ $\beta^{fl/fl}$; α -Cre* and control retinas a small subset of dRGCs positive for the
426 transcription factors *Tbr2* and *Foxp2*, markers for non-image-forming RGCs and DS-
427 RGCs respectively (Nadal-Nicolás et al., 2014). Together with our transcriptomic data
428 (upregulation of genes such as *Cartpt* (expressed in DS-RGCs)), these results strongly
429 suggest that the large number of dRGCs in *Gsk3 $\alpha^{fl/+}$ $\beta^{fl/fl}$; α -Cre* retina might indeed be
430 DS-RGCs projecting into the MTN. It has been recently proposed that dRGCs might
431 be also involved in predator detection by integrating overhead visual information
432 (Nadal-Nicolás et al., 2014). Using suitable and complementary visual tests, our
433 genetic model with an excess of dRGCs could be highly valuable to complete the
434 functional identification of the dRGCs in vision.

435 Overall, our results demonstrate the critical role of GSK3 in tightly regulating the
436 number of a rare type of dRGCs, poorly described yet. Therefore, *Gsk3* mutant mice,
437 encompassing a large number of dRGCs in their retina, offer a unique and powerful
438 model system to further study the embryonic origin, synaptic connections and visual
439 function of dRGCs in mammals.

440

441 **MATERIALS AND METHODS**

442 **Animals and tissue collection**

443 All animal experiments have been carried out in accordance with the European
444 Communities Council Directive of 22 September 2010 (2010/63/EEC), European
445 Union guidelines effective and with the Association for Research in Vision and
446 Ophthalmology statement for the use of animals in ophthalmic and visual Research.
447 All animal care and experimentation were also conducted in accordance with
448 guidelines, under the license APAFIS#1018-2016072611404304 by the Institutional
449 animal care committee n°059 in France and by Animal Care and Use Committee at the
450 National Institutes of Health (ASP#650). *Gsk3 α* and *Gsk3 β* floxed mice were
451 generously provided by Dr. Jim Woodgett (University of Toronto, Canada). Floxed
452 *Gsk3* mice were mated with those carrying the retina-specific regulatory element of
453 murine *Pax6* driving the expression of the Cre recombinase (*α -Cre*) in retinal
454 progenitors as early as E10.5 generously provided by Peter Gruss (Marquardt et al.,
455 2001). Mice from either sex were used for experimental procedures. All mouse
456 genotyping was performed as described (Roger et al., 2010).

457

458 **Hematoxylin & eosin (H&E) staining, and immunostaining**

459 Methacrylate sections were used for H&E staining as previously described (Roger et
460 al., 2012). For IHC on frozen sections, enucleated eyeballs were fixed at the required
461 stage in 4% PFA for 60 min on ice and incubated in an increasing concentration of
462 sucrose (10%, 20% and 30%), then embedded in OCT. Embedded eyeballs were
463 serially cut to 12 μ m sections using a cryostat. For embryonic stages, pregnant females
464 were euthanized and whole heads of pups were harvested in paraffin. IHC was
465 performed as described (Hamon et al., 2019). Primary and secondary antibodies are
466 listed in Supplementary Table 1. Sections were counterstained with 1:1000 4',6-
467 diamidino-2-phenylindole (DAPI) (1 mg/mL, Thermo Scientific).

468

469 **EdU labeling and TUNEL assay**

470 For EdU labelling, females were injected intraperitoneally with 10 mM of 5-ethynyl-20-
471 deoxyuridine (EdU) (Life Technology). EdU incorporation was detected on paraffin
472 sections or frozen sections using the Click-iT EdU Imaging Kit following manufacturer's
473 recommendations (Life Technology). Apoptosis was detected by terminal
474 deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)
475 assays using *in situ* cell death detection kit (Promega). All images were acquired using
476 a Zeiss LSM710 confocal microscope and Zen software (Zeiss).

477

478 **Immunoblotting**

479 Frozen retinas were lysed by sonication in lysis buffer (20 mM Na₂HPO₄, 250 mM
480 NaCl, 30 mM NaPPi, 0.1% NP40, 5mM EDTA, 5mM DTT) supplemented with protease
481 inhibitor cocktail (Sigma-Aldrich). Lysates concentration was determined using a Lowry
482 protein assay kit (Bio-Rad) following sonication and centrifugation. Protein
483 supernatants were separated under denaturing condition by SDS-PAGE, transferred

484 onto nitrocellulose membrane and probed with antibodies (listed in Supplementary
485 Table 1.), as described (Hamon et al., 2019). Proteins were visualized using enhanced
486 chemiluminescence kit (Bio-Rad). α -tubulin was used for normalization. Quantification
487 was performed using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public
488 domain at NIH).

489

490 **Retinal flat mount**

491 Fixed retinas were permeabilized and blocked in a solution containing 0.5% Triton-
492 X100, 5% donkey normal serum, 1XPBS, 0.1 g/L thimerosal for 1 day at RT under
493 agitation. Primary antibodies were diluted in a solution containing 0.5% Triton-X100,
494 5% donkey normal serum, 10% Dimethyl Sulfoxide, 1XPBS, 0.1 g/L thimerosal for 3
495 days at RT under agitation. The retinas were then washed for 1 day in PBST (1XPBS,
496 0.5% Triton-X100). Secondary antibodies were diluted in the same solution as primary
497 antibodies and left for 2 days. After washing retinas for 1 day, they were mounted on
498 slides and imaged using a scanning confocal microscope (Olympus, FV1000). Primary
499 and secondary antibodies are listed in Supplementary Table 1.

500 **Electroretinography**

501 Electroretinogram (ERG) recordings were performed using a focal ERG module
502 attached to Micron IV (Phoenix Research Laboratory). Briefly, mice were dark-adapted
503 overnight and prepared for the experiment under dim-red light. The mice were
504 anesthetized with ketamine (100 mg/kg) and xylazine (10mg/kg) and received topical
505 proparacaine hydrochloride (0.5%, Alcon) via eye drops. Pupils were dilated with
506 tropicamide (1%, Alcon) and phenylephrine (2.5%, Alcon) and lightly coated with
507 GONAK hypromellose ophthalmic demulcent solution (2.5%, Akorn). Lens of the
508 Micron IV was placed directly on the cornea, and a reference electrode was placed on
509 the mouse head. Scotopic responses were elicited with a series of flashes of increasing
510 light intensities from -1.7 to 2.2 cd.s/m². Photopic responses were elicited under rod-
511 desensitizing background light with a series of flashes of increasing light intensities
512 from -0.5 to 2.8 cd.s/m². Values of a- and b-wave were extracted and plotted for
513 comparisons between groups of interest.

514

515 **Optomotor response (OMR)**

516 Real time video tracking and automated measurements of compensatory head
517 movements in freely moving mice were performed using an OMR recording setup
518 (Kretschmer et al., 2013, 2015) (Phenosys, Berlin, Germany). Each mouse was placed
519 on a platform in the center of four computer-controlled LCD monitors. Visual stimuli
520 were sinusoidally modulated luminance gratings generated by four LCD screens (60
521 Hz refresh rate; OkrArena, PhenoSys GmbH, Berlin, Germany), presented with a
522 constant rotation. Video tracking considered the animal's distance to the monitors,
523 thereby keeping the spatial frequency of the retinal image constant and providing data
524 for automated OMR quantifications.

525 OMRs were recorded using two different Michelson contrasts and different spatial
526 frequencies (presented in random order) in the two mouse groups: 100% contrast or
527 50% contrast (n=13 for *Gsk3 α ^{fl/+} β ^{fl/fl}* and 18 for *Gsk3 α ^{fl/+} β ^{fl/fl}; α -Cre* genotype). All stimuli
528 were presented for 60 s randomly in either clockwise or counterclockwise direction.

529 The measurements were completed in three trials for each animal. At 100% and 50%
530 contrast, OMRs were recorded in response to sinusoidal gratings at 12 spatial
531 frequencies between 0.0125 and 0.5 cycles per degree (cpd). The number of head
532 movements recorded at a speed range from 2 to 14 degrees per second in the same
533 direction as the stimulus (T_Correct) and in the opposite direction (T_Incorrect) were
534 used to calculate the OMR indices ($T_Correct / T_Incorrect$) at each spatial frequency.
535

536 **Retrograde labeling of retinal ganglion cells**

537 For retrograde labeling, eyes were enucleated with a piece of the optic nerve and fixed
538 in 4% PFA for 30 min. Rhodamine B isothiocyanate–Dextran (Sigma-Aldrich) was
539 applied on the top of the optic nerve and incubated for 60 min. Eyes were flat mounted
540 after the remaining dye was washed out for 48 hours in PBS at 4°C. Z series images
541 were acquired using SP5 confocal microscope (Leica Biosystems), and 3D
542 reconstruction was performed using Volocity (Perkin Elmer).
543

544 **Anterograde labeling of retinal ganglion cell projections**

545 Anterograde labeling

546 For anterograde tracing of retinal projections, a Cholera Toxin beta subunit (CTB) was
547 used. Animal were anesthetized using a cocktail of ketamine (60 mg/kg) and xylazine
548 (10 mg/kg) and a subsequent bilateral injection of 1.2uL CTB at 1mg/ml coupled to
549 either an Alexa-555 or -647 (Lifesciences) were performed intravitreally. Three days
550 following the injection, mice were perfused with 4% PFA.

551 Tissue Clearing and 3D imaging

552 For 3D imaging of CTB-labeled brains, a methanol clearing protocol was carried out
553 using modification from the iDISCO+ protocol (Belle et al., 2014, 2017). Briefly, brains
554 were dehydrated by immersion in progressive baths of methanol/1X PBS (20%, 40%,
555 60%, 80%, 100%, 100%) for 2 hours each at RT on a tube rotator (SB3, Stuart) at
556 14rpm, using a 15 mL centrifuge tube (TPP, Dutcher) protected from light. Following
557 these baths, samples were immersed overnight in 2/3 Dichloromethane (DCM; Sigma-
558 Aldrich) and then a 30-min bath in 100 % DCM before being transferred in Di-Benzyl
559 Ether (DBE; Sigma-Aldrich) overnight prior imaging.

560 3D imaging/Image acquisition for all samples was performed as previously described
561 (Dobin et al., 2013). Acquisitions were done using an ultramicroscope I (LaVision
562 Biotec) with the ImspectorPro software (LaVision Biotec). The step size between each
563 image was fixed at 2 μm with a numerical aperture of 0.120 and 150ms acquisition
564 using a PCO Edge SCMOS CCD camera (2,560 x 2,160 pixel size, LaVision BioTec).

565 Image analysis

566 Imaris x64 software (Version9.1.2, Bitplane) was used for all image analysis. Stack
567 images were first converted from .tiff to .ims files using the Imaris file converter v9.1.2.
568 3D reconstruction was visualized with the “volume rendering” function. To isolate ipsi-
569 and contralateral MTN volumes, manual segmentation was carried out using the
570 “surface” tool and the isoline selection (density, 10%). Each ipsi- and contra-lateral
571 projection of the MTN was segmented to generate a volume (μm^3). Movie

572 reconstruction with .tiff series were done with ImageJ (1.50e, Java 1.8.0_60, 64-bit)
573 and iMovie (version 10.1.1).

574 **Whole transcriptome sequencing and data analysis**

575 Whole transcriptome analysis was performed on three independent biological
576 replicates from *Gsk3 $\alpha^{fl/+}$ $\beta^{fl/fl};\alpha$ -Cre* and *Gsk3 $\alpha^{fl/+}$ $\beta^{fl/fl}$* retina at P60. After harvesting, both
577 retinas for each animal were immediately frozen. RNA was extracted using Nucleospin
578 RNA Plus kit (Macherey-Nagel). RNA quality and quantity were evaluated using a
579 BioAnalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies). Stranded RNA-Seq
580 libraries were constructed from 100 ng high-quality total RNA (RIN > 8) using the
581 TruSeq Stranded mRNA Library Preparation Kit (Illumina). Paired-end sequencing of
582 40 bases length was performed on a NextSeq 500 system (Illumina). Pass-filtered
583 reads were mapped using STAR and aligned to mouse reference genome GRCm38.94
584 (Dobin et al., 2013). Count table of the gene features was obtained using
585 FeatureCounts (Liao et al., 2014). Normalization, differential expression analysis and
586 FPKM (fragments per kilobase of exon per million fragments mapped) values were
587 computed using EdgeR (Chen et al., 2015). An FPKM filtering cutoff of 1 in at least one
588 of the 6 samples was applied. A False Discovery Rate (FDR) of less than or equal to
589 0.05 was considered significant and a fold change cutoff of 1.5 was applied to identify
590 differentially expressed genes. Comprehensive gene list analysis, enriched biological
591 pathways, gene annotation, were based on Gene Ontology classification system using
592 Metascape (Zhou et al., 2019). Data visualization was done using GOplot R package
593 (Walter et al., 2015). To evaluate the expression of the DEGs in RGCs, we used
594 published whole transcriptome analysis from purified RGCs available on Gene
595 Expression Omnibus database (GSE87647) (Sajgo et al., 2017).

596

597 **Gene expression analysis by Real-Time PCR (RT-qPCR)**

598 After RNA extraction using Nucleospin RNA Plus kit (Macherey-Nagel), 500 ng of total
599 RNA was reverse transcribed using the iScript cDNA Synthesis Kit according to
600 manufacturer instructions (BioRad). Primers used for RT-qPCR are shown in
601 Supplementary Table 2. For each RT-qPCR, 2 μ L of a ten-fold dilution of synthesized
602 cDNA was used, and the reactions were performed in technical triplicates on a C1000
603 thermal cycler (CFX96 real-time system, BioRad) using SsoFast EvaGreen Supermix
604 (BioRad) as previously described (Livak and Schmittgen, 2001). RT-qPCR
605 experiments were performed on three independent biological replicates. Differential
606 expression was determined using the $\Delta\Delta$ Ct method with the geometric average of
607 *Rps26* and *Srp72* as endogenous controls (Livak and Schmittgen, 2001).

608

609 **Statistical analysis**

610 Statistical analysis was performed with GraphPad Prism 8.3.0 (GraphPad Software,
611 La Jolla California USA). Results are reported as mean \pm SEM. Nonparametric Mann-
612 Whitney U test was used to analyze cell counting and qPCR data. *P* value \leq 0.05 was
613 considered significant. For OMR assay statistical analysis, a Grubbs test was
614 performed at 5% to remove outliers followed by 2-way ANOVA (genotype and spatial
615 frequency) with Bonferroni Post Hoc test. *P* value \leq 0.05 was considered significant.

616

617 **ACKNOWLEDGEMENT**

618 We are grateful to Dr. Jim Woodgett for providing the *Gsk3* floxed lines and Tudor
619 Badea and Peter Gruss for the alpha-Cre line. We would like also to thank Leah
620 Thomas, Elodie-Kim Grellier, Yide Mi, and Jessica Gumerson for their help with mouse
621 colonies and technical support. This research was supported by the CNRS, Retina
622 France and by the Intramural Research Program of the National Eye Institute
623 (EY000450 and EY000546). EK was supported by the Ernst Ludwig Ehrlich
624 Studienwerk.

625

626 **AUTHOR'S CONTRIBUTION**

627 E.K., R.J.V., C.H., designed and performed the experiments and analyzed the data,
628 S.L. AND P.S. performed the experiments and analyzed the data, A.C. designed the
629 experiments, analyzed the data and revised the manuscript, M.P., A.S., J.E.R.
630 designed the study, analyzed the data, wrote the manuscript with the help of E.K. R.J.V
631 and C.H. J.E.R supervised the study.

632

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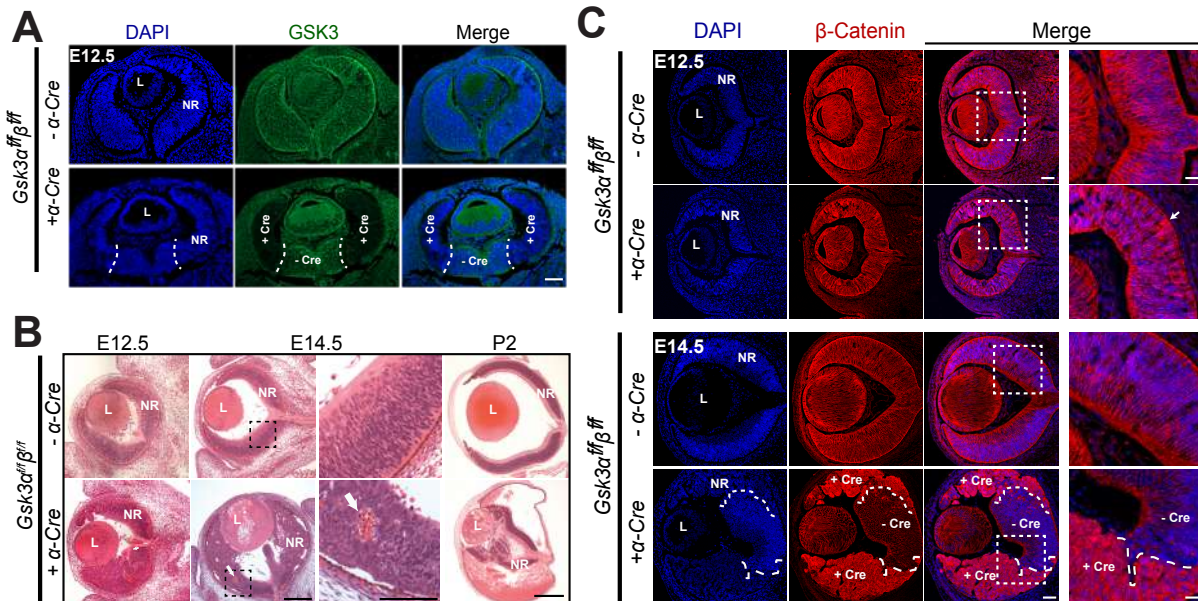
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831 **FIGURES AND FIGURE LEGENDS**

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836 **Figure 1. Developmental defects and microphthalmia in *Gsk3*-deficient retina**
837 **with aberrant nuclear translocation of β -catenin, a key effector of the Wnt**
838 **canonical pathway.** (A) Immunohistochemistry (IHC) of E12.5 retina from *Gsk3 $\alpha^{fl/fl};\beta^{fl/fl}$*
839 mice expressing or not α -Cre using a pan-GSK3 antibody (green) shows efficient
840 deletion at the periphery where the Cre expression has been previously reported
841 (delimited by dashed-line) Scale bar: 100 μ m. (B) Hematoxylin and eosin (H&E)
842 staining on methacrylate sections at E12.5, E14.5 and P2 reveals large retinal
843 morphogenesis defects in *Gsk3 $\alpha^{fl/fl};\beta^{fl/fl};\alpha$ -Cre* with blood invasion into the eyeball
844 (showed by white arrow). L, Lens; NR, neural retina. Scale bar: 100 μ m at E12.5 and
845 E14.5. 500 μ m at P2. For the magnification of P14.5, scale bar: 50 μ m. (C) β -catenin
846 accumulation (red) at E14.5 in the Cre-expressing area of *Gsk3 $\alpha^{fl/fl};\beta^{fl/fl};\alpha$ -Cre*
847 animals. White arrow indicates a small area of accumulation. Magnification on the right panel
848 shows the squared delimited area of β -catenin accumulation. L, Lens; NR, neural
849 retina; + Cre, Cre-expressing area; - Cre, area without Cre expression Scale bar:
850 100 μ m, magnification area scale bar: 40 μ m.

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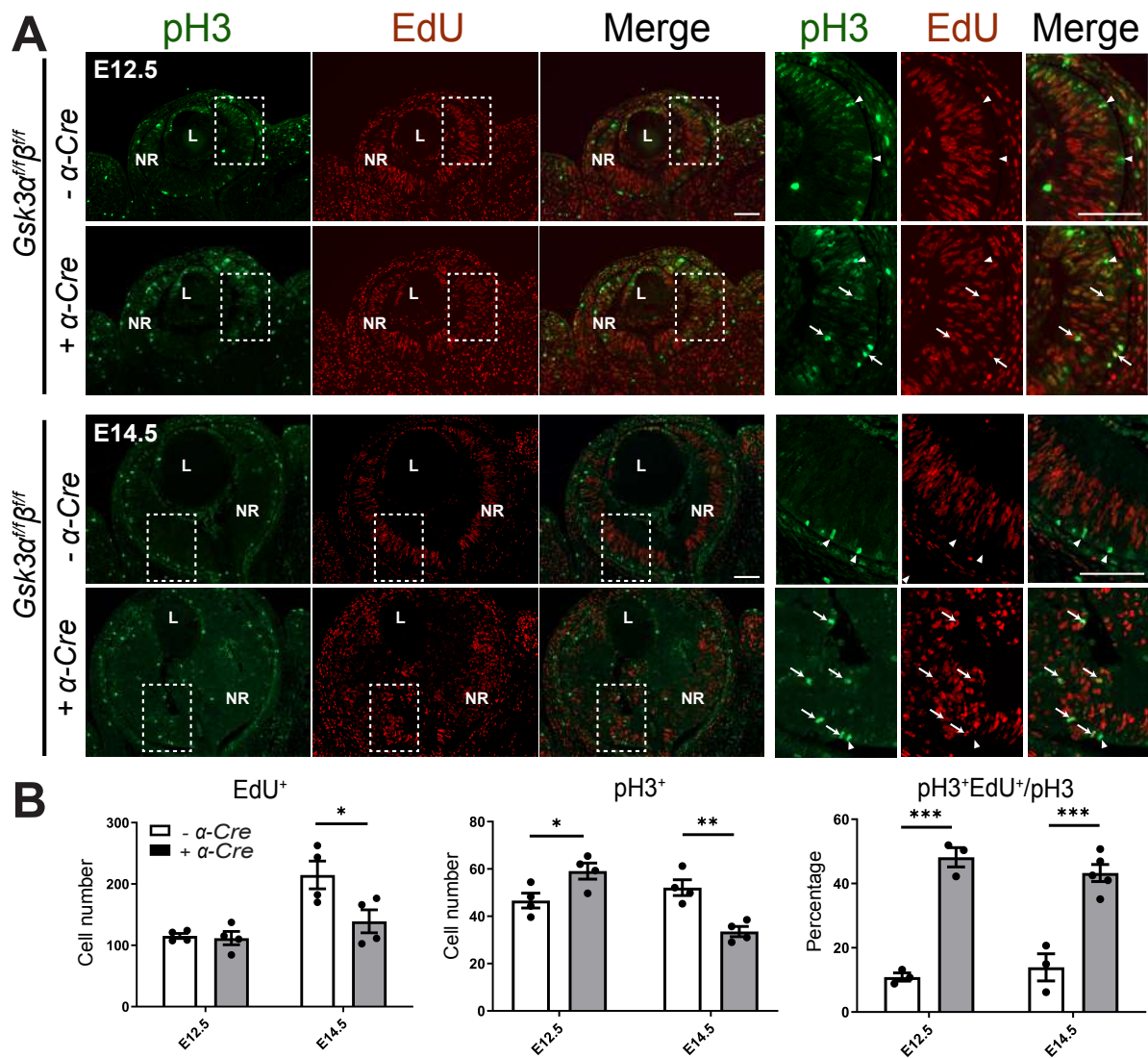
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863 **Figure 2. Lack of GSK3 signaling alters cell cycle progression of retinal**

864 **progenitor cells.** (A) Mislocalization of pH3-positive cells in the neuroblastic layer of

865 *Gsk3α^{fl/fl};α-Cre* retina. E12.5 and E14.5 retina stained for pH3 (late-G2/M-phase,

866 green) and EdU (marker of S-phase, red) following a 16H chase. Magnification on the

867 right panel shows the squared delimited area. Arrowheads, pH3-positive and EdU-

868 negative cells; Arrows, pH3- and EdU-positive cells. Scale bar: 100 μm, magnification

869 area scale bar: 100 μm. (B) Lack of GSK3 signaling alters cell cycle progression of

870 retinal progenitors. Quantification at E12.5 and E14.5 of the number of EdU-positive

871 cells (left panel), pH3-positive cells (middle panel) and double positive cells for EdU

872 and pH3 among pH3-positive cells (right panel) in *Gsk3α^{fl/fl};α-Cre* animals and

873 controls. Mean ± SEM values are presented from 3 to 5 independent retinas for each

874 genotype, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$.

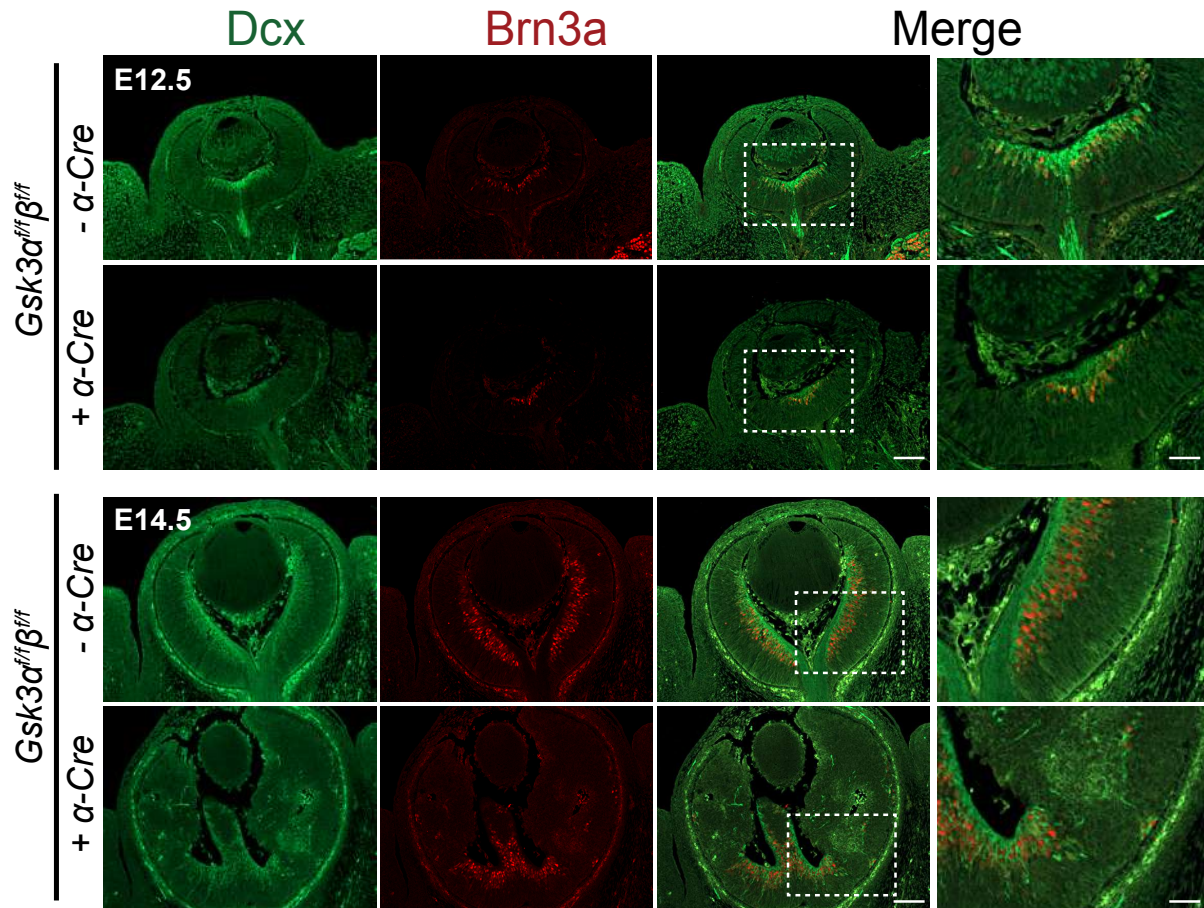
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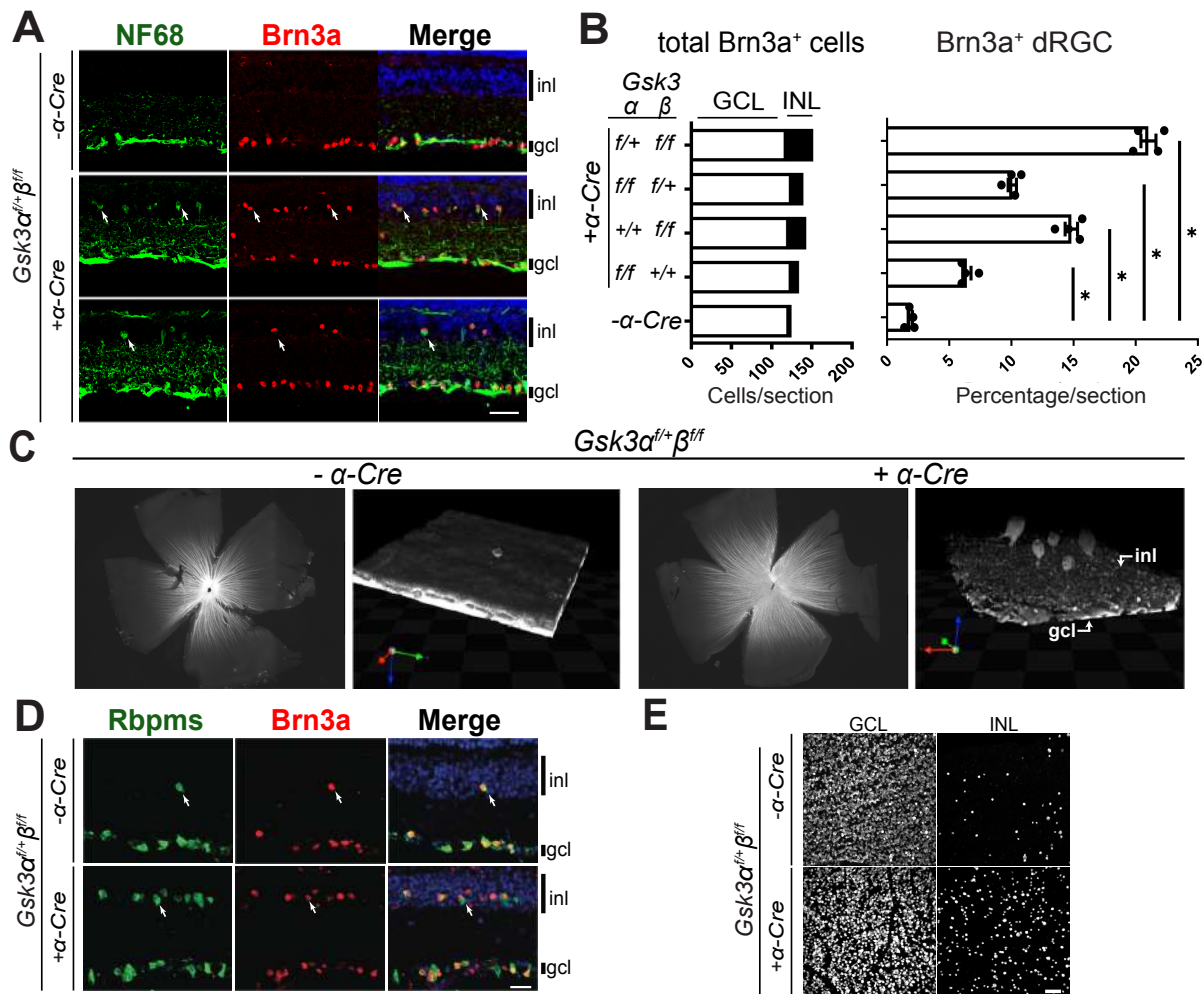
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882 **Figure 3. Lack of *Gsk3 α* and *Gsk3 β* expression in retinal progenitors blocks**
883 **retinal progenitor differentiation.** IHC using Doublecortin (Dcx) (green) and Brn3a
884 (red) antibodies shows the localization of neuronal precursor cells and RGCs,
885 respectively, at the central and basal part of the neuroblastic layer of E12.5 control
886 retina. In contrast, neural differentiation is not observed in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* retina, with
887 the exception of a small central area where Cre is not expressed. At E14.5, Dcx- and
888 Brn3a-positive cells are distributed across the whole retina in controls, whereas they
889 are absent in periphery in *Gsk3 $\alpha^{ff}\beta^{ff};\alpha$ -Cre* mice. Magnification on the right-hand side
890 shows the squared delimited area and depicts the boundary between a Cre-positive
891 and a Cre-negative area. Scale bar: 100 μ m, magnification 40 μ m.

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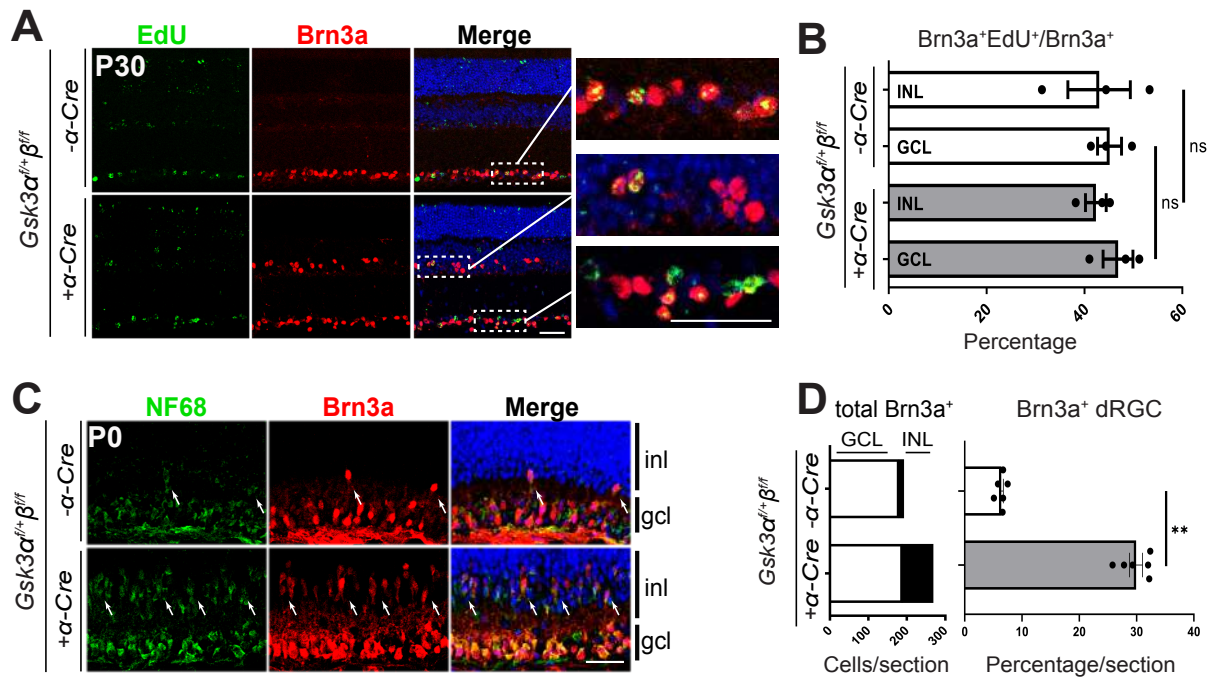
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Figure 4. Gradual loss of $Gsk3\alpha$ and/or $Gsk3\beta$ leads to an increased number of Brn3a-positive retinal ganglion cells displaced in the inner nuclear layer (INL) of adult retina. (A) Brn3a (red) and NF68 (green) IHC on 2-month-old $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha-Cre$ mouse retina reveals the presence of supernumerary displaced retinal ganglion cells (dRGCs, arrows) in the INL of $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha-Cre$ compared to littermate controls. Top panel represents control retinas, middle panel a peripheral retinal area, and bottom panel a more central area. Scale bar: 20 μm . (B) Gradual loss of $Gsk3\alpha$ and $Gsk3\beta$ alleles ($Gsk3\alpha^{f/f}\beta^{+/+}$, $Gsk3\alpha^{+/+}\beta^{f/f}$, $Gsk3\alpha^{f/+}\beta^{f/f}$ or $Gsk3\alpha^{f/f}\beta^{f/+}$) leads to a gradual increase of Brn3a-positive RGCs located to the INL, with the highest number observed in $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha-Cre$ animals. Left stacked histogram represents counting of the total number of Brn3a-positive cells per section located in the GCL (white bars) and in the INL (black bars). Right histogram represents the percentage of the dRGCs among the total number of Brn3a-positive cells per section for each combination. Mean \pm SEM values are presented from 4 biological replicates, * indicates $P \leq 0.05$. (C) dRGCs send their axons into the optic nerve. Visualization of dRGCs after 3D reconstruction of 2-month-old flat mounted retina of control and $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha-Cre$ animals following retrograde labeling with Rhodamin-Dextran applied onto the optic nerve. inl: inner nuclear layer, gcl: ganglion cell layer. (D) Brn3a (red) and Rbpms (green) IHC on 2-month-old mouse retina reveal the co-expression of these two RGC markers (dRGCs, arrows) in the INL of both $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha-Cre$ dRGCs and in littermate controls. Scale

925 bar: 20 μm . (E) Flat mounted retina from $Gsk3\alpha^{f/+}\beta^{ff}$; α -Cre and littermate controls
926 labelled with anti-Rbpms antibody demonstrated the large number of Rbpms-positive
927 dRGCs in the INL of $Gsk3\alpha^{f/+}\beta^{ff}$; α -Cre mice.

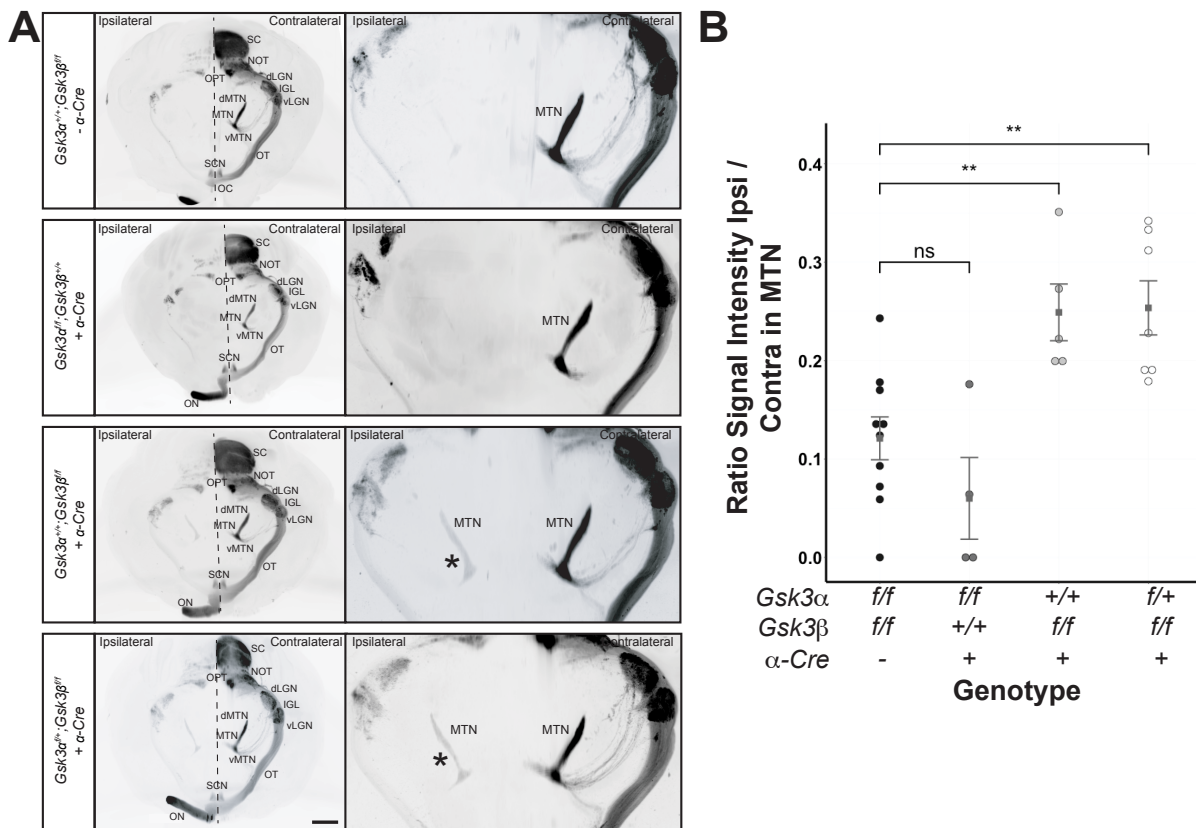
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972 **Figure 5. dRGCs are produced in the same differentiation wave as oRGC located**
 973 **in the GCL.** (A) EdU- (green) and Brn3a-positive cells (red) were found both in the
 974 GCL and in the INL of 30-days old $Gsk3\alpha^{f/+}\beta^{ff}; \alpha-Cre$ animals after a single injection of
 975 EdU at E12.5. (B) Percentage of EdU- and Brn3a-positive cells located either in the
 976 GCL or in the INL among total number of Brn3a-positive cells. Mean \pm SEM values are
 977 presented from 3-4 biological replicates, ns: not significant (C) Brn3a (red) and NF68
 978 (green) immunostaining on P0 mouse retina revealed that a large number of dRGCs
 979 were already present in $Gsk3\alpha^{f/+}\beta^{ff}; \alpha-Cre$ but they were fewer in littermate controls
 980 (white arrows). (D) Left stacked histogram represents counting of the total number of
 981 Brn3a-positive cells per section located in the GCL (white bars) and in the INL (black
 982 bars) of $Gsk3\alpha^{f/+}\beta^{ff}; \alpha-Cre$ retina. Right histogram represents the percentage of the
 983 dRGCs among the total number of Brn3a-positive cells per section. Mean \pm SEM
 984 values are presented from 6 biological replicates, ** indicates $P \leq 0.01$. inl, inner
 985 nuclear layer; gcl, ganglion cell layer. Scale bar: 20 μ m.

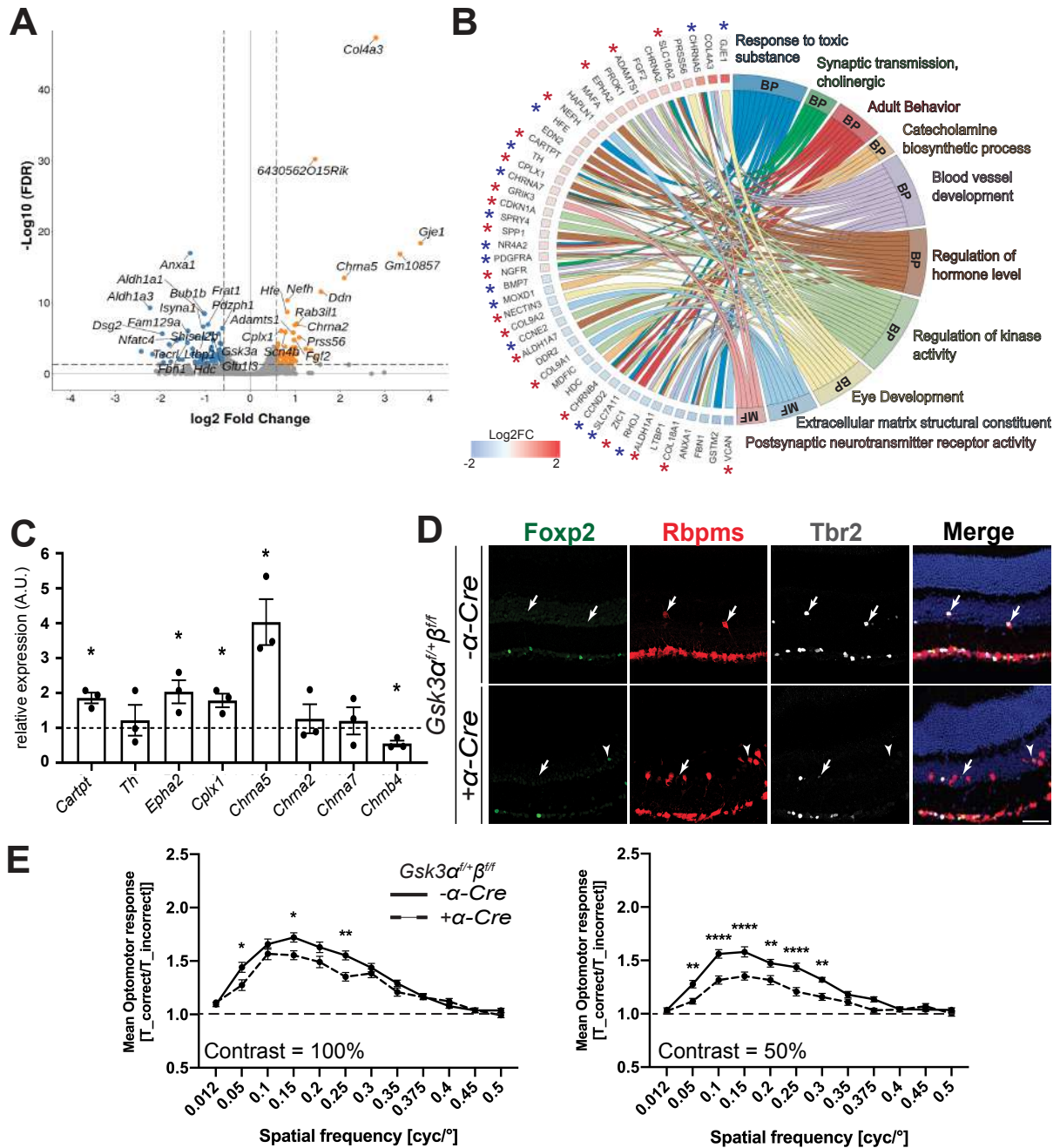
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1001 **Figure 6. Lack of *Gsk3β* results in RGC projections into the ipsilateral Medial**
 1002 **Terminal Nucleus.** (A) All panels are light sheet fluorescence microscopy of solvent-
 1003 cleared adult brain from control, *Gsk3α*^{f/f} β ^{+/+}; α -Cre, *Gsk3α*^{+/+} β ^{f/f}; α -Cre and *Gsk3α*^{f/+} β ^{f/f};
 1004 α -Cre animals after intravitreal injection of CTB coupled to either an Alexa-555 or -647.
 1005 Ipsilateral projections of RGCs into the MTN was observed in the absence of *Gsk3β*
 1006 expression. SC, superior colliculus; NOT, nucleus of optic tract; dLGN, dorsal lateral
 1007 geniculate nucleus; vLGN, ventral lateral geniculate nucleus; IGL, intergeniculate
 1008 leaflet; OPT, Olivary Pretectal Nucleus; dMTN, dorsal medial terminal nucleus; MTN,
 1009 medial terminal nucleus; vMTN, ventral medial terminal nucleus; OT, Optic tract; SCN,
 1010 suprachiasmatic nucleus; ON, optic nerve. Scale bar: 1mm; * indicates the ipsilateral
 1011 MTN. (B) Quantification of the signal intensity ratio between ipsilateral and
 1012 contralateral MTN in controls and *Gsk3* mutants (including *Gsk3α*^{f/f} β ^{+/+}; α -Cre,
 1013 *Gsk3α*^{+/+} β ^{f/f}; α -Cre, and *Gsk3α*^{f/+} β ^{f/f}; α -Cre). ns: non-significant, ** $P \leq 0.01$.

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1028 **Figure 7. dRGC characterization in $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha\text{-Cre}$ retina.** (A) Volcano plot

1029 representation of differentially expressed genes between $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha\text{-Cre}$ and

1030 control retina plotted on the x-axis (\log_2 scale). FDR adjusted significance is plotted

1031 on the y-axis. Orange and blue dots: significantly up-regulated and down-regulated

1032 genes in $Gsk3\alpha^{f/+}\beta^{f/f}; \alpha\text{-Cre}$ retina, respectively. Vertical dashed lines represent

1033 $FC=1.5$. Horizontal dashed line represents $FDR=0.05$. (B) Chord plot representation of

1034 DEGs related to GO annotations belonging to either molecular functions (MF) or

1035 biological process (BP). Overlaps in GO annotation amongst genes within each

1036 category are visualized. * correspond to genes expressed in previously published

1037 purified RGCs (blue, slightly expressed genes in RGCs between 1 and 5 FPKM; red,

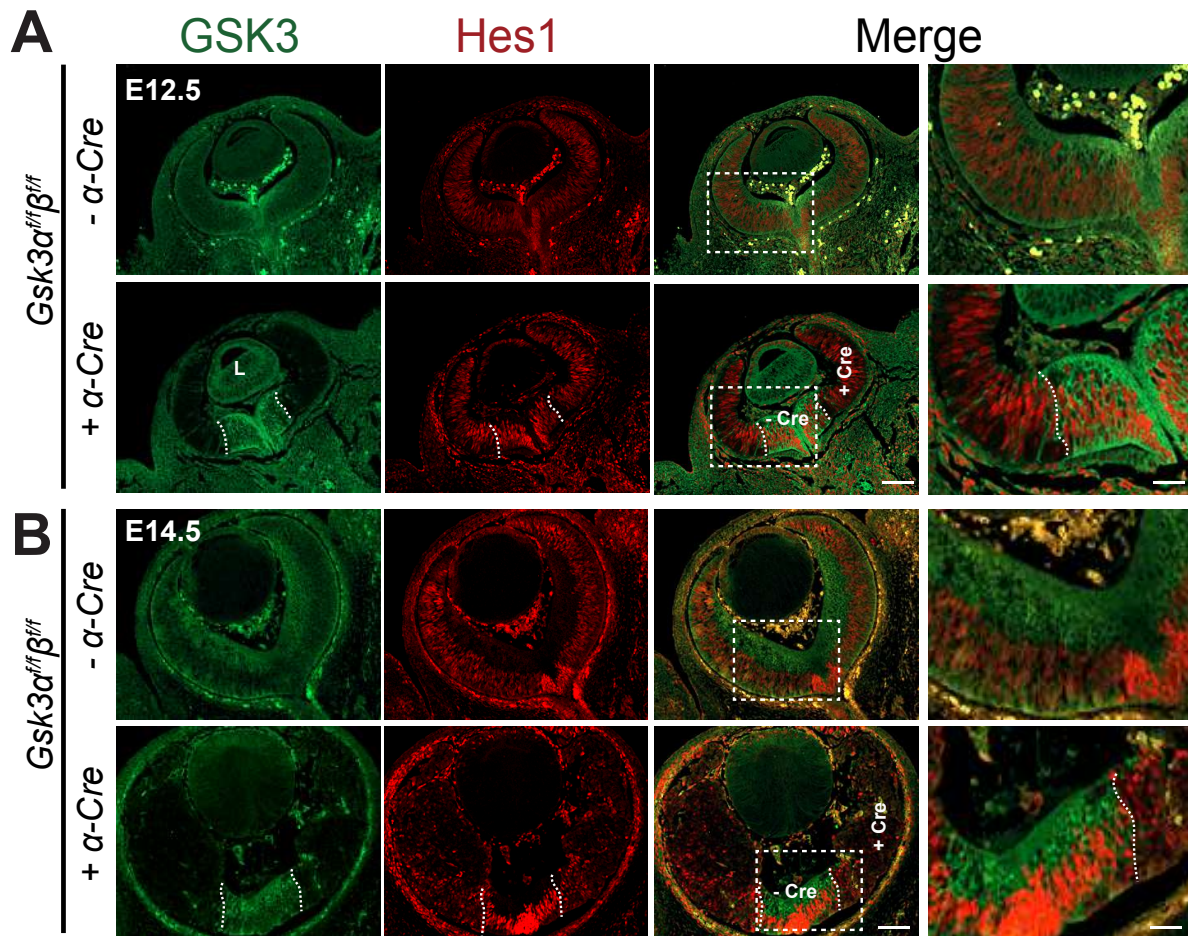
1038 highly expressed genes in RGCs more than 5 FPKM). (C) RT-qPCR validation of

selected DEGs identified by RNA-seq analysis. Differential expression analysis by RT-

1039 qPCR of *Cartpt*, *Th*, *Epha2*, *Cplx1*, *Chrna5*, *Chrna2*, *Chrna7*, *Chrn4* in *Gsk3 α ^{fl/+} β ^{fl/fl}; α -*
1040 *Cre* retina at 2-months of age, relative to littermate control retina levels. All values are
1041 expressed as the Mean \pm SEM from three biological replicates. * indicates $P \leq 0.05$.
1042 (D) IHC on 2-month-old mouse retina reveals the presence of a subset of dRGCs
1043 (Rbpms-positive dRGCs, red) in *Gsk3 α ^{fl/+} β ^{fl/fl}; α -*Cre* expressing either the transcription
1044 factor *Tbr2* (grey) or *Foxp2* (green). Arrows indicate *Tbr2* and Rbpms-positive dRGCs;
1045 arrowheads represent *Foxp2* and Rbpms-positive dRGCs. Scale bar: 50 μ m. (E) The
1046 mean OMR indices (\pm SEM) are plotted as a function of spatial frequency for each
1047 genotype (n=13 for *Gsk3 α ^{fl/+} β ^{fl/fl} and 18 for *Gsk3 α ^{fl/+} β ^{fl/fl}; α -*Cre* genotype). The baseline
1048 (1; dashed line) represents unspecific head movements and no response to the
1049 stimulus. OMR at 100% and 50% contrast in *Gsk3 α ^{fl/+} β ^{fl/fl}; α -*Cre* mice (dashed line) and
1050 controls (black line). * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$.
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1054 SUPPLEMENTARY FIGURE LEGENDS

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1058 **Supplementary data 1. Lack of *Gsk3α* and *Gsk3β* expression impairs the**
1059 **maintenance of the pool of retinal progenitors.** IHC on E12.5 and E14.5 $Gsk3\alpha^{ff/\beta^{ff}}$
1060 and $Gsk3\alpha^{ff/\beta^{ff}};\alpha$ -Cre retina using Hes1 (red) and pan-GSK3 (green) indicates a loss
1061 of retinal progenitors at E14.5. Magnification on the right panel shows the squared
1062 delimited area. Dashed lines delimit the central *Gsk3*-expressing area and the *Gsk3*-
1063 deleted area at the periphery Scale bar: 100 μ m, magnification area scale bar 40 μ m.

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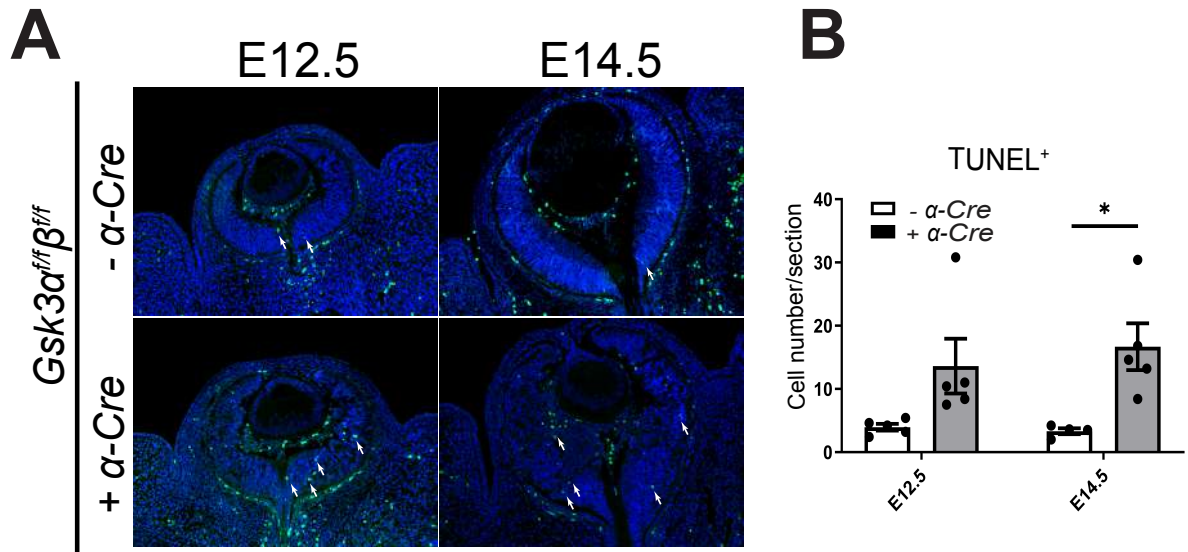
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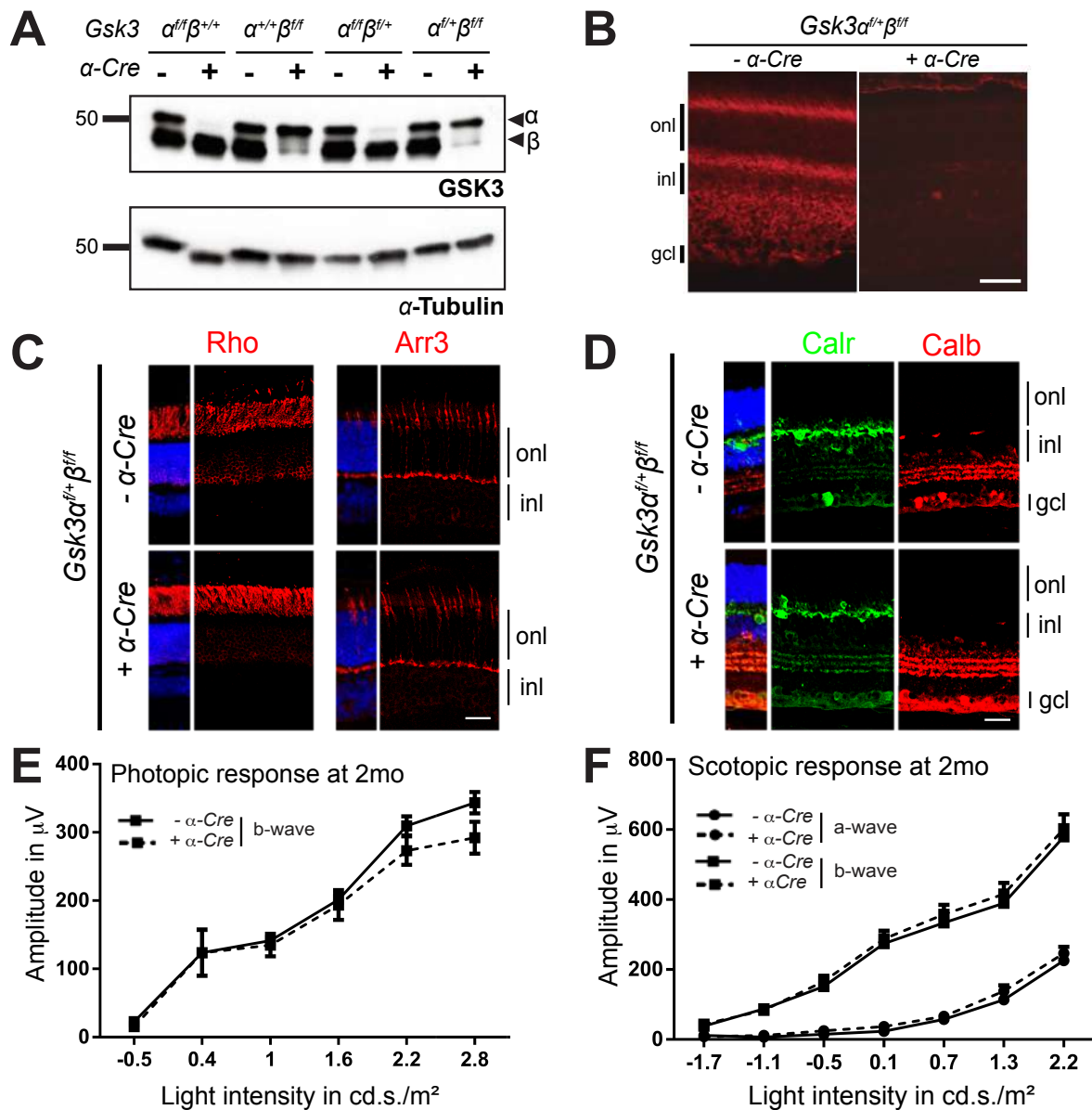
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1078 **Supplementary data 2. Lack of *Gsk3 α* and *Gsk3 β* expression in retinal**
1079 **progenitors leads to increased cell death.** (A) TUNEL assay on E12.5 and E14.5
1080 *Gsk3 $\alpha^{ff/ff}$; $\beta^{ff/ff}$; α -Cre* and control animals reveals an increase of cell death (TUNEL-
1081 positive cells, green) in *Gsk3 $\alpha^{ff/ff}$; $\beta^{ff/ff}$; α -Cre* animals compared to littermate controls. (B)
1082 Quantification of the number of TUNEL-positive cells per retinal section. Mean \pm SEM
1083 values are presented from 5 to 6 biological replicates for E12.5, 4-5 biological
1084 replicates for E14.5, * indicates $P \leq 0.05$.

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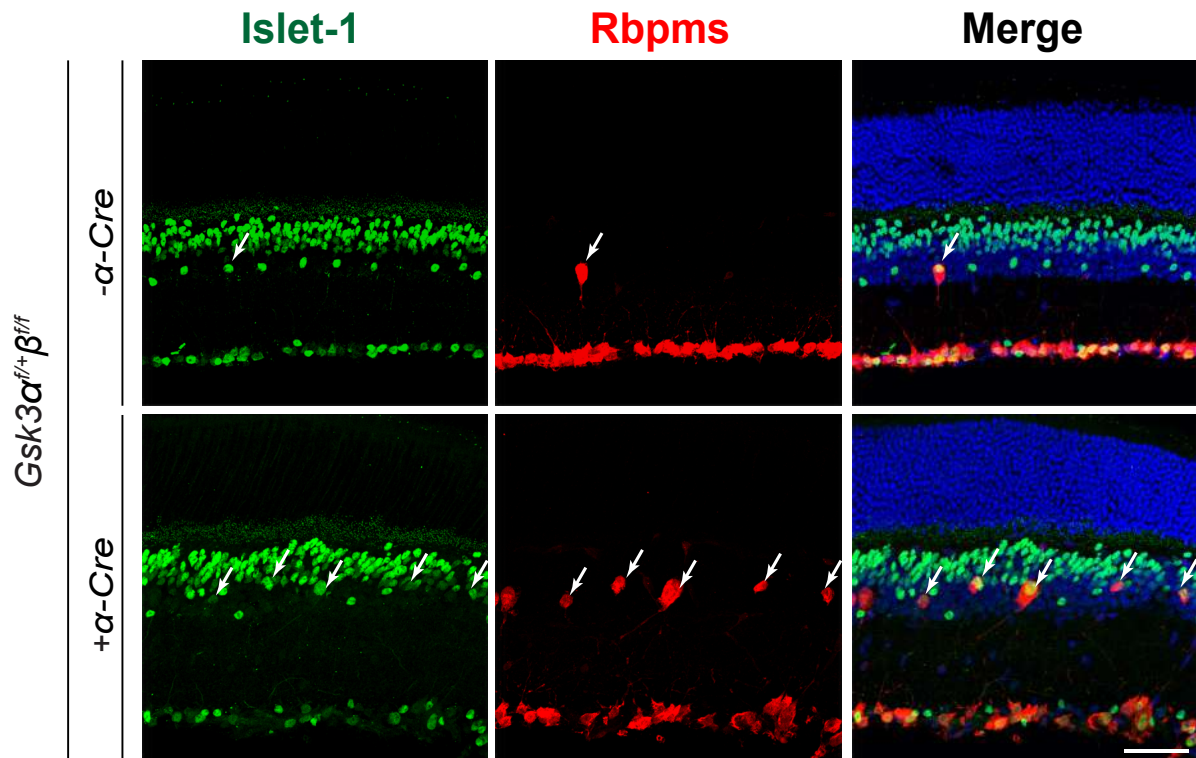


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1109 **Supplementary data 3. One allele of either *Gsk3α* or *Gsk3β* is sufficient for the**
 1110 **development of a functional retina.** (A) Immunoblot analysis of protein extracts from
 1111 2-month-old animals with different combination of *Gsk3α* and *Gsk3β* floxed alleles
 1112 (*Gsk3α^{fl/fl}β^{+/+}*, *Gsk3α^{+/+}β^{fl/fl}*, *Gsk3α^{fl/+}β^{fl/fl}* or *Gsk3α^{fl/fl}β^{fl/+}*) with or without *Cre*
 1113 recombinaise using anti-panGSK3 antibody (recognizing both isoforms) reveals decreased
 1114 expression of GSK3α or GSK3β (arrowheads). α -Tubulin is used as loading control.
 1115 (B) IHC on 2-month-old retinal sections from control and *Gsk3α^{fl/+}β^{fl/fl}*, α -*Cre* retinas with
 1116 or without *Cre* recombinaise using anti-GSK3β antibody (red) showing ubiquitous
 1117 *Gsk3β* expression in all retinal layers, whereas its expression is lost in the *Cre*-
 1118 expressing retina. (C) Expression of only one *Gsk3* allele (*Gsk3α*) is sufficient for
 1119 proper photoreceptor development. IHC using anti-Rhodopsin (Rho, red) and anti-
 1120 Cone arrestin (Arr3, red) antibodies to label rod and cone photoreceptors, respectively.
 1121 (D) Expression of only one *Gsk3* allele (*Gsk3α*) is sufficient for proper interneuron
 1122 development. IHC using anti-Calretinin (Calr, green) and anti-Calbindin (Calb, red)

1123 antibodies to label horizontal and amacrine cells, respectively. (B-D) onl, outer nuclear
1124 layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 20 μ m. (E, F)
1125 Electroretinogram (ERG) recording in 2-month-old *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* animals and
1126 littermate controls. Photopic (cones) (E) and scotopic (rods) (F) response in
1127 *Gsk3 $\alpha^{f/+}$ $\beta^{f/f}$; α -Cre* animals are similar to controls. Mean \pm SEM intensity response
1128 curves of a- and b-wave responses averaged from 8 biological replicates of each
1129 genotype.

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Supplementary data 4. dRGCs express the nuclear factor Islet-1. IHC on 2-month-old mouse retina reveals that most dRGCs (Rbpms-positive dRGCs, white arrows, red) in the INL of $Gsk3\alpha^{fl/+}\beta^{fl/fl}; \alpha-Cre$ and littermate controls were positive for Islet-1 (green), a marker expressed in the nuclei of ganglion cells, and of cholinergic amacrine cells, ON-bipolar cells, and subpopulations of horizontal cells. Scale bar: 50 μ m.

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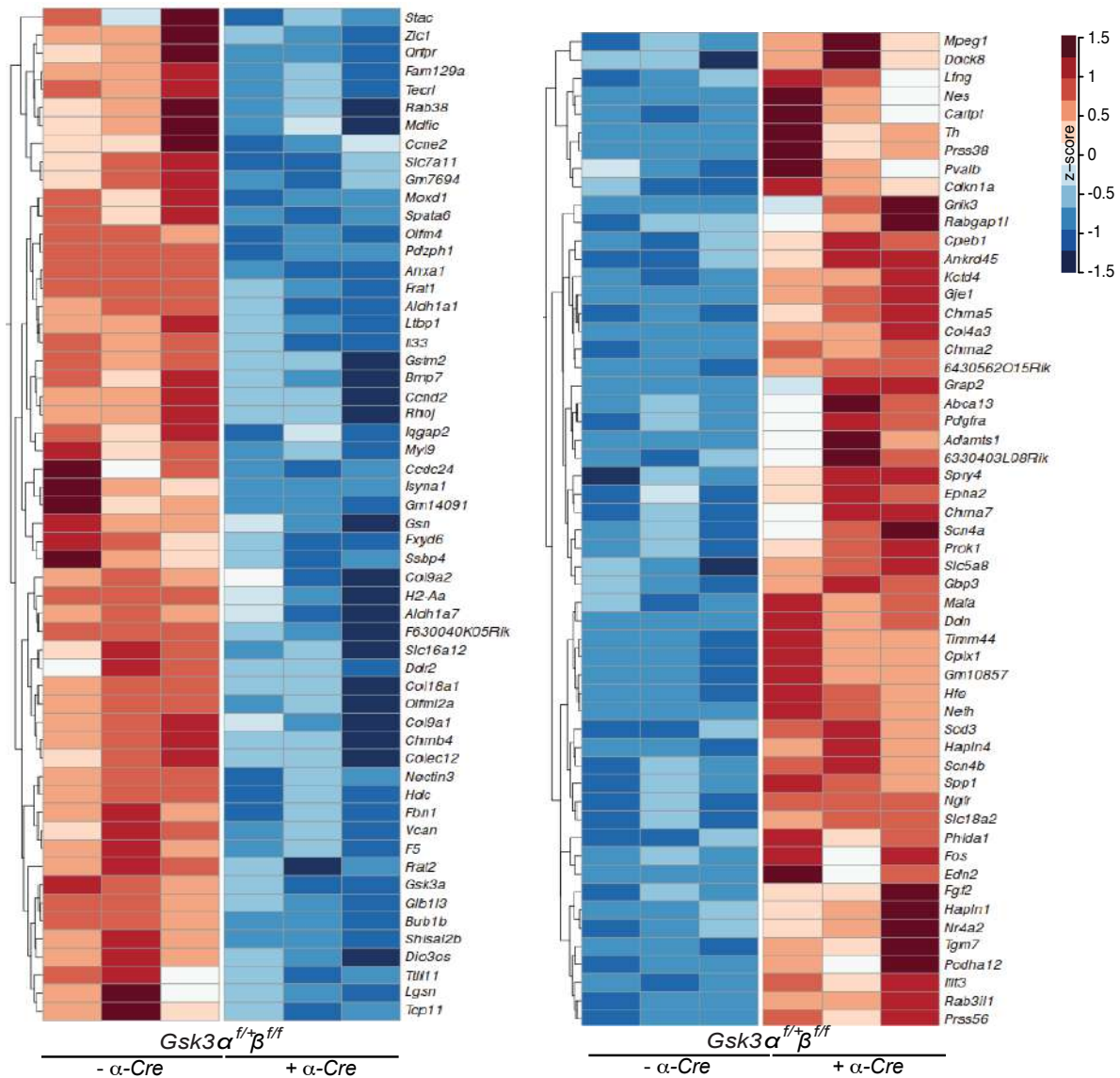
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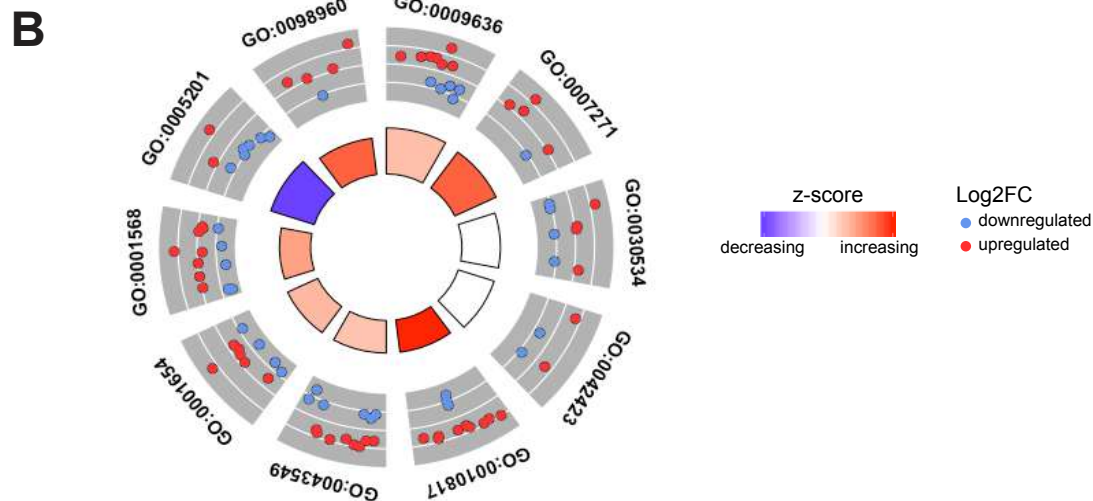
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Supplementary data 5. Hierarchical clustering of the identified differentially expressed genes. Hierarchical clustering representing the 111 DEGs ($abs(FC)\geq 1.5$; $FDR\leq 0.05$; $FPKM>1$) between 2-month-old $Gsk3\alpha^{f/+}\beta^{f/f}$; $\alpha-Cre$ retina and littermate control were clustered by their Z-score. Left panel corresponds to downregulated genes; Right panel corresponds to upregulated genes.

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Category	ID	Term	Count	List Total	Genes	FDR
BP	GO:0009636	response to toxic substance	12	354	Chrna7, Aldh1a1, Gstm2, Anxa1, Nr4a2, Th, Slc7a11, Chrb4, Chrna5, Chrna2, Slc18a2, Nefh	1.72E-03
BP	GO:0007271	synaptic transmission, cholinergic	5	34	Chrna7, Ngfr, Chrb4, Chrna5, Chrna2	2.38E-03
BP	GO:0030534	adult behavior	8	193	Chrna7, Ccnd2, Nr4a2, Zic1, Slc7a11, Cartpt, Chrb4, Chrna5	9.01E-03
BP	GO:0042423	catecholamine biosynthetic process	4	26	Hdc, Nr4a2, Th, Moxd1	1.03E-02
BP	GO:0010817	regulation of hormone levels	12	574	Chrna7, Aldh1a1, Cplx1, Edn2, Hfe, Anxa1, Pdgfra, Spp1, Aldh1a7, Cartpt, Slc18a2, Mafa	2.18E-02
BP	GO:0043549	regulation of kinase activity	14	774	Chrna7, Bmp7, Ccnd2, Ccne2, Cdkn1a, Epha2, Fbn1, Fgf2, Mdfic, Ddr2, Pdgfra, Spry4, Cartpt, Prok1	2.18E-02
BP	GO:0001654	eye development	10	402	Aldh1a1, Bmp7, Epha2, Fgf2, Pdgfra, Th, Nectin3, Prss56, Gje1, Rhoj	2.26E-02
BP	GO:0001568	blood vessel development	13	731	Chrna7, Adams1, Bmp7, Col18a1, Col4a3, Epha2, Fgf2, Anxa1, Ngfr, Pdgfra, Rhoj, Prok1, Ltbp1	3.46E-02
KEGG	mmu04151	PI3K-Akt signaling pathway	11	346	Ccnd2, Ccne2, Cdkn1a, Col4a3, Col9a1, Col9a2, Epha2, Fgf2, Ngfr, Pdgfra, Spp1	2.69E-03
MF	GO:0005201	extracellular matrix structural constituent	8	143	Col18a1, Col4a3, Col9a1, Col9a2, Hapln1, Vcan, Fbn1, Ltbp1	2.38E-03
MF	GO:0098960	postsynaptic neurotransmitter receptor activity	5	58	Chrna7, Grik3, Chrb4, Chrna5, Chrna2	1.03E-02
RGS	R-MMU-622327	Postsynaptic nicotinic acetylcholine receptors	4	13	Chrna7, Chrb4, Chrna5, Chrna2	2.08E-03
RGS	R-MMU-1474244	Extracellular matrix organization	10	266	Bmp7, Col18a1, Col4a3, Col9a1, Hapln1, Fbn1, Fgf2, Ddr2, Spp1, Ltbp1	2.38E-03
TRRUST	TRR00919	Regulated by: Egr1	5	71	Chrna7, Ccnd2, Fgf2, Ngfr, Th	2.18E-02
TRRUST	TRR00820	Regulated by: Atf4	3	13	Cdkn1a, Fgf2, Ddr2	2.26E-02
TRRUST	TRR01387	Regulated by: Rbpj	3	18	Cdkn1a, Fos, Lfng	4.45E-02



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1215 **Supplementary data 6. Identification of enriched pathways from DEGs identified**
 1216 **in 2-month-old *Gsk3a^{fl/+}β^{fl/fl}; α-Cre* retina.** (A) Gene ontology (GO) annotations of
 1217 DEGs in *Gsk3a^{fl/+}β^{fl/fl}; α-Cre* retina compared to littermate controls. Top over-
 1218 represented pathways for Biological process (BP), Molecular Function (MF), KEGG
 1219 (Kyoto Encyclopedia of Genes and Genomes) and TRRUST (Transcriptional
 1220 Regulatory Relationships Unrevealed by Sentence-based Text mining) were identified
 1221 by enrichment analysis using Metascape. (B) Circular visualization for BP and MF of
 1222 GO enrichment analysis. Down-regulated genes (blue dots) and up-regulated genes
 1223 (red dots) within each GO pathway are plotted based on logFC. Z-score bars indicate
 1224 if an entire GO category is more likely to be increased or decreased based on the
 1225 genes within it.

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1233 **Supplementary Table 1. List of primary and secondary antibodies used for**
 1234 **immunohistochemistry (IF) and western blot (WB)**

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 1236 **Primary antibodies**

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
α -tubulin	mouse	SIGMA	T5168		1:200.000
GSK3 α/β	mouse	Thermo Fisher Scientific	44-610	1:250	1:1000
GSK3 β	mouse	BD	610201	1:250	
β -catenin	rabbit	Abcam	ab2365	1:200	
HES1	rabbit	Cell Signaling	11988S	1:200	
Brn3a	mouse	Santa Cruz	sc-8429	1:200	
GFP	goat	Abcam	ab290-50	1:500	
pH3	rabbit	EMD Millipore	06-570	1:300	
Calbindin D-28k	rabbit	Swant	300	1:100	
Calretinin	mouse	EMD Millipore	MAB1568	1:1000	
Cone Arrestin	rabbit	EMD Millipore	AB15282	1:1000	
Rhodopsin	Mouse	Abcam	MAB5316	1:2000	
Tbr2	Rat	Ebioscience	14-4876	1 :300	
Foxp2	Goat	Santa Cruz	sc-21069	1 :1000	
Rbpms	Rabbit	PhosphoSolutions	1830-RBPMS	1 :400	

1237 **Secondary antibodies**

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
Alexa Fluor 555 anti-mouse IgG2A	goat	Thermo Fisher Scientific	A21127	1:1000	
Alexa Fluor 555 anti-mouse IgG2B	goat	Thermo Fisher Scientific	A21147	1:1000	
Alexa Fluor 488 anti-rabbit	donkey	Thermo Fisher Scientific	A21206	1:1000	
Alexa Fluor 488 anti-mouse IgG1	goat	Thermo Fisher Scientific	A21240	1:1000	
Alexa Fluor 488 anti-rabbit	goat	Thermo Fisher Scientific	A21244	1:1000	
HRP anti-mouse IgG	goat	Sigma-Aldrich	A4416		1:5000

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1240 **Supplementary Table 2. List of primers used for RT-qPCR**

Gene name	Primer F	Primer R
<i>Cartpt</i>	TAAAGTTTTCGTTCCCTCAG	CAACACCATTCGAGGCATTCT
<i>Th</i>	ACTATGCCTCTCGTATCCAGC	CGGATGGTGTGAGGACTGTC
<i>Epha2</i>	GACCTCCCCATCTTCATTTGG	GCGTACAGTGCCCTAGTCATA
<i>Cplx1</i>	GGTGATGAGGAAAAGGACCCC	TCTTGCGTACTTTGCTTTGC
<i>Chrna5</i>	CTTGAGTACCAACACTGTCCG	CCAGTACTCCAAGATGCCCT
<i>Chrna2</i>	CATTATCGTCTGCTTCCTGGG	CTTGGAGCCAACATGAGGGA
<i>Chrna7</i>	CTGTAGCTGTCGGTCTTGAGA	CAATGATATGCCGGTGATGGG
<i>Chrn4</i>	AAACTGATCTGGCTACCTCCC	GTAGAGAGTCCAGGAGATGCC

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