



**HAL**  
open science

## Glycogen Synthase Kinase 3 regulates the genesis of displaced retinal ganglion cells

Elena Kisseleff, Robin J Vigouroux, Catherine Hottin, Sophie Lourdel, Leah Thomas, Parth Shah, Alain Chédotal, Muriel Perron, Anand Swaroop, Jerome E Roger

► **To cite this version:**

Elena Kisseleff, Robin J Vigouroux, Catherine Hottin, Sophie Lourdel, Leah Thomas, et al.. Glycogen Synthase Kinase 3 regulates the genesis of displaced retinal ganglion cells. *eNeuro*, 2021, 8 (3), pp.ENEURO.0171-21.2021. 10.1523/ENEURO.0171-21.2021 . hal-03354295

**HAL Id: hal-03354295**

**<https://hal.sorbonne-universite.fr/hal-03354295>**

Submitted on 24 Sep 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

---

*Research Article: New Research | Sensory and Motor Systems*

## **Glycogen Synthase Kinase 3 regulates the genesis of displaced retinal ganglion cells**

<https://doi.org/10.1523/ENEURO.0171-21.2021>

**Cite as:** eNeuro 2021; 10.1523/ENEURO.0171-21.2021

Received: 19 April 2021

Revised: 19 August 2021

Accepted: 2 September 2021

---

*This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.*

**Alerts:** Sign up at [www.eneuro.org/alerts](http://www.eneuro.org/alerts) to receive customized email alerts when the fully formatted version of this article is published.

Copyright © 2021 Kisseleff et al.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license, which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

---

1 **Glycogen Synthase Kinase 3 regulates the genesis of displaced**  
2 **retinal ganglion cells.**

3

4 Elena Kisseleff<sup>1</sup>, Robin J Vigouroux<sup>3</sup>, Catherine Hottin<sup>1</sup>, Sophie Lourdel<sup>1</sup>, Leah  
5 Thomas<sup>1</sup>, Parth Shah<sup>2</sup>, Alain Chédotal<sup>3</sup>, Muriel Perron<sup>1\*</sup>, Anand Swaroop<sup>2\*</sup> and  
6 Jerome E Roger<sup>1,2\*</sup>

7

8 <sup>1</sup> Paris-Saclay Institute of Neuroscience, CERTO-Retina France, CNRS, Université  
9 Paris-Saclay, Orsay 91405, France

10 <sup>2</sup> Neurobiology-Neurodegeneration and Repair Laboratory, National Eye Institute,  
11 National Institutes of Health, Bethesda, MD, USA

12 <sup>3</sup> Sorbonne Universités, UPMC Université Paris 06, INSERM, CNRS, Institut de la  
13 Vision, 75012 Paris, France

14

15 Running Title: **GSK3 controls displaced retinal ganglion cell genesis in the**  
16 **retina**

17

18 \*Corresponding authors:

19 – Jerome E Roger. [jerome.roger@universite-paris-saclay.fr](mailto:jerome.roger@universite-paris-saclay.fr)

20 – Anand Swaroop. [swaropa@nei.nih.gov](mailto:swaropa@nei.nih.gov)

21 – Muriel Perron. [muriel.perron@universite-paris-saclay.fr](mailto:muriel.perron@universite-paris-saclay.fr)

22

23

24

25

26

27 **ABSTRACT**

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

44

45

---

46 **Significance Statement**

47 Glycogen Synthase Kinase 3 (GSK) proteins ( $Gsk3\alpha$  or  $Gsk3\beta$ ) are key mediators of  
48 signaling pathways, especially in the central nervous system but poorly described in  
49 the retina. Here we show that the complete loss of GSK3 in mouse retinal progenitors  
50 leads to microphthalmia. However, when only one allele of  $Gsk3\alpha$  or  $Gsk3\beta$  are  
51 present, all cell types are present with a functional retina. More importantly, we  
52 unexpectedly uncover a unique role of GSK3 in controlling the genesis of retinal  
53 ganglion cells in the inner nuclear layer which could correspond to a rare and poorly  
54 characterized retinal cell type. Therefore, our mouse models potentially offer a unique  
55 and powerful model system to study the visual function of dRGCs in mammals.

56

57

---

58 **INTRODUCTION**

59 Glycogen Synthase Kinase 3 alpha (GSK3 $\alpha$ ) and beta (GSK3 $\beta$ ) are  
60 functionally redundant serine/threonine kinases encoded by two different genes,  
61 sharing 95% identity in their kinase domain (Doble et al., 2007). GSK3 exists at the  
62 crossroads of multiple signaling pathways and acts as a key molecular switch to  
63 mediate their output and guide distinct cellular processes (Cole, 2012; Doble and  
64 Woodgett, 2003; Espinosa et al., 2003; Jin et al., 2009; Shimizu et al., 2008; Wang  
65 and Li, 2006). Among the signaling pathways regulated by GSK3 kinases, Wnt  
66 canonical pathway is the most well described, with GSK3 $\beta$  inhibition triggering an  
67 increase in  $\beta$ -catenin protein levels and its nuclear translocation to activate target  
68 gene expression (Doble and Woodgett, 2003).

69 GSK3 is a key regulator of neural stem/precursor cell proliferation in  
70 developing as well as adult brain (Eom and Jope, 2009; Hur and Zhou, 2010; Kim et  
71 al., 2009; Pachenari et al., 2017). Conditional deletion and gain of function  
72 experiments indicate that GSK3 promotes neuronal differentiation (Hur and Zhou,  
73 2010; Kim et al., 2009). GSK3 exerts its effects through phosphorylation of key  
74 proteins involved in neural development, including proneural factors such as  
75 Neurogenin 2 and NeuroD (Li et al., 2012; Moore et al., 2002). In addition, GSK3  
76 fine-tunes the balance between cell death and survival, and its altered function is  
77 associated with neurodegenerative pathologies including Alzheimer's disease,  
78 bipolar disorders, and Parkinson's disease (Golpich et al., 2015; Jacobs et al., 2012;  
79 Kremer, 2011; Li et al., 2014; Maurer et al., 2014; Medina et al., 2011).

80 GSK3 kinases are widely expressed in the developing retina (Pérezleón et al.,  
81 2013). GSK3-dependent phosphorylation is shown to control the timing of proneural  
82 factor activity and thereby regulate retinal cell fate determination. For instance,  
83 inhibition of GSK3 signaling in the developing *Xenopus* retina leads to increase in

---

84 early-born cell types at the expense of late-born cells (Marcus et al., 1998; Moore et  
85 al., 2002).

86 To elucidate GSK3 function in mammalian retina development, we generated  
87 conditional loss-of-function alleles of *Gsk3 $\alpha$*  and *Gsk3 $\beta$*  in retinal progenitor cells. We  
88 show that complete loss of both GSK3 kinases severely impacts retinal morphology  
89 with microphthalmia phenotype, which could be completely rescued with the  
90 expression of just one *Gsk3 $\alpha$*  or *Gsk3 $\beta$*  wild-type allele. We also noted the presence  
91 of a large number of displaced retinal ganglion cells (dRGCs) in the inner nuclear  
92 layer in the absence of either *Gsk3 $\alpha$*  or *Gsk3 $\beta$* . In normal conditions, this is a rare  
93 retinal cell subtype, poorly characterized so far. Anterograde labeling of the axonal  
94 ganglion cell projections into the brain of *Gsk3* mutant mice, allowed us to further  
95 support their dRGCs identity. Our study thus identifies GSK3 as a possible  
96 determinant of dRGC genesis. We also provide transcriptomic data and visual tests  
97 suggesting that at least a subset of these supernumerary dRGCs in *Gsk3* mutant  
98 retinas are direction-selective RGCs.

99

## 100 **MATERIALS AND METHODS**

### 101 **Animals and tissue collection**

102 All animal experiments have been carried out in accordance with the European  
103 Communities Council Directive of 22 September 2010 (2010/63/EEC), European  
104 Union guidelines effective and with the Association for Research in Vision and  
105 Ophthalmology statement for the use of animals in ophthalmic and visual Research.  
106 All animal care and experimentation were also conducted in accordance with  
107 guidelines, under the license APAFIS#1018-2016072611404304 by the Institutional  
108 animal care committee n°059 in France and by Animal Care and Use Committee at

---

109 the National Institutes of Health (ASP#650). *Gsk3 $\alpha$*  and *Gsk3 $\beta$*  floxed mice were  
110 generously provided by Dr. Jim Woodgett (University of Toronto, Canada). Floxed  
111 *Gsk3* mice were mated with those carrying the retina-specific regulatory element of  
112 murine *Pax6* driving the expression of the Cre recombinase ( *$\alpha$ -Cre*) in retinal  
113 progenitors as early as E10.5 generously provided by Peter Gruss (Roger et al.,  
114 2012). Mice are on a mixed background C57Bl6/J and 129/SvJ. Animals from either  
115 sex were used for experimental procedures. All mouse genotyping was performed as  
116 described (Hamon et al., 2019).

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

118 Methacrylate sections were used for H&E staining as previously described (Hamon et  
119 al., 2019). For IHC on frozen sections, enucleated eyeballs were fixed at the required  
120 stage in 4% PFA for 60 min on ice and incubated in an increasing concentration of  
121 sucrose (10%, 20% and 30%), then embedded in OCT. Embedded eyeballs were  
122 serially cut to 12  $\mu$ m sections using a cryostat. For embryonic stages, pregnant  
123 females were euthanized and whole heads of pups were harvested in paraffin. IHC  
124 was performed as described (Kretschmer et al., 2015, 2013). Primary and secondary  
125 antibodies are listed in Table 1. Sections were counterstained with 1:1000 4',6-  
126 diamidino-2-phenylindole (DAPI) (1 mg/mL, Thermo Scientific).

### 127 **EdU labeling and TUNEL assay**

128 For EdU labelling, females were injected intraperitoneally with 10 mM of 5-ethynyl-  
129 20-deoxyuridine (EdU) (Life Technology). EdU incorporation was detected on paraffin  
130 sections or frozen sections using the Click-iT EdU Imaging Kit following  
131 manufacturer's recommendations (Life Technology). Apoptosis was detected by  
132 terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling  
133 (TUNEL) assays using *in situ* cell death detection kit (Promega). All images were  
134 acquired using a Zeiss LSM710 confocal microscope and Zen software (Zeiss).

---

**135 Immunoblotting**

136 Frozen retinas were lysed by sonication in lysis buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 250 mM  
137 NaCl, 30 mM NaPPi, 0.1% NP40, 5mM EDTA, 5mM DTT) supplemented with  
138 protease inhibitor cocktail (Sigma-Aldrich). Lysates concentration was determined  
139 using a Lowry protein assay kit (Bio-Rad) following sonication and centrifugation.  
140 Protein supernatants were separated under denaturing condition by SDS-PAGE,  
141 transferred onto nitrocellulose membrane and probed with antibodies (listed in Table  
142 1), as described (Belle et al., 2017, 2014). Proteins were visualized using enhanced  
143 chemiluminescence kit (Bio-Rad).  $\alpha$ -tubulin was used for normalization.  
144 Quantification was performed using ImageJ software (<http://imagej.nih.gov/ij/>;  
145 provided in the public domain at NIH).

**146 Retinal flat mount**

147 Fixed retinas were permeabilized and blocked in a solution containing 0.5% Triton-  
148 X100, 5% donkey normal serum, 1XPBS, 0.1 g/L thimerosal for 1 day at RT under  
149 agitation. Primary antibodies were diluted in a solution containing 0.5% Triton-X100,  
150 5% donkey normal serum, 10% Dimethyl Sulfoxide, 1XPBS, 0.1 g/L thimerosal for 3  
151 days at RT under agitation. The retinas were then washed for 1 day in PBST  
152 (1XPBS, 0.5% Triton-X100). Secondary antibodies were diluted in the same solution  
153 as primary antibodies and left for 2 days. After washing retinas for 1 day, they were  
154 mounted on slides and imaged using a scanning confocal microscope (Olympus,  
155 FV1000). Primary and secondary antibodies are listed in Table 1.

**156 Electroretinography**

157 Electroretinogram (ERG) recordings were performed using a focal ERG module  
158 attached to Micron IV (Phoenix Research Laboratory). Briefly, mice were dark-  
159 adapted overnight and prepared for the experiment under dim-red light. The mice  
160 were anesthetized with ketamine (100 mg/kg) and xylazine (10mg/kg) and received

---

161 topical proparacaine hydrochloride (0.5%, Alcon) via eye drops. Pupils were dilated  
162 with tropicamide (1%, Alcon) and phenylephrine (2.5%, Alcon) and lightly coated with  
163 GONAK hypromellose ophthalmic demulcent solution (2.5%, Akorn). Lens of the  
164 Micron IV was placed directly on the cornea, and a reference electrode was placed  
165 on the mouse head. Scotopic responses were elicited with a series of flashes of  
166 increasing light intensities from -1.7 to 2.2 cd.s/m<sup>2</sup>. Photopic responses were elicited  
167 under rod-desensitizing background light with a series of flashes of increasing light  
168 intensities from -0.5 to 2.8 cd.s/m<sup>2</sup>. Values of a- and b-wave were extracted and  
169 plotted for comparisons between groups of interest.

#### 170 **Optomotor response (OMR)**

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

180 OMRs were recorded using two different Michelson contrasts and different spatial  
181 frequencies (presented in random order) in the two mouse groups: 100% contrast or  
182 50% contrast (n=13 for *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>* and 18 for *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* genotype). All stimuli  
183 were presented for 60 s randomly in either clockwise or counterclockwise direction.  
184 The measurements were completed in three trials for each animal. At 100% and  
185 50% contrast, OMRs were recorded in response to sinusoidal gratings at 12 spatial  
186 frequencies between 0.0125 and 0.5 cycles per degree (cpd). The number of head

---

187 movements recorded at a speed range from 2 to 14 degrees per second in the same  
188 direction as the stimulus (T\_Correct) and in the opposite direction (T\_Incorrect) were  
189 used to calculate the OMR indices (T\_Correct / T\_Incorrect) at each spatial  
190 frequency.

### 191 **Retrograde labeling of retinal ganglion cells**

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

### 198 **Anterograde labeling of retinal ganglion cell projections**

#### 199 Anterograde labeling

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

#### 205 Tissue Clearing and 3D imaging

206 For 3D imaging of CTB-labeled brains, a methanol clearing protocol was carried out  
207 using modification from the iDISCO+ protocol (Dobin et al., 2013). Briefly, brains  
208 were dehydrated by immersion in progressive baths of methanol/1X PBS (20%, 40%,  
209 60%, 80%, 100%, 100%) for 2 hours each at RT on a tube rotator (SB3, Stuart) at  
210 14rpm, using a 15 mL centrifuge tube (TPP, Dutcher) protected from light. Following  
211 these baths, samples were immersed overnight in 2/3 Dichloromethane (DCM;

---

212 Sigma-Aldrich) and then a 30-min bath in 100 % DCM before being transferred in Di-  
213 Benzyl Ether (DBE; Sigma-Aldrich) overnight prior imaging.

214 3D imaging/Image acquisition for all samples was performed as previously described  
215 (Liao et al., 2014). Acquisitions were done using an ultramicroscope I (LaVision  
216 Biotec) with the InspectorPro software (LaVision Biotec). The step size between  
217 each image was fixed at 2  $\mu\text{m}$  with a numerical aperture of 0.120 and 150ms  
218 acquisition using a PCO Edge SCMOS CCD camera (2,560 x 2,160 pixel size,  
219 LaVision BioTec).

#### 220 Image analysis

221 Imaris x64 software (Version9.1.2, Bitplane) was used for all image analysis. Stack  
222 images were first converted from .tiff to .ims files using the Imaris file converter  
223 v9.1.2. 3D reconstruction was visualized with the “volume rendering” function. To  
224 isolate ipsi- and contralateral MTN volumes, manual segmentation was carried out  
225 using the “surface” tool and the isoline selection (density, 10%). Each ipsi- and  
226 contra-lateral projection of the MTN was segmented to generate a volume ( $\mu\text{m}^3$ ).  
227 Movie reconstruction with .tiff series were done with ImageJ (1.50e, Java 1.8.0\_60,  
228 64-bit) and iMovie (version 10.1.1).

#### 229 **Whole transcriptome sequencing and data analysis**

230 Whole transcriptome analysis was performed on three independent biological  
231 replicates from  $Gsk3\alpha^{fl/+}\beta^{fl/+};\alpha\text{-Cre}$  and  $Gsk3\alpha^{fl/+}\beta^{fl/fl}$  retina at P60. After harvesting, both  
232 retinas for each animal were immediately frozen. RNA was extracted using  
233 Nucleospin RNA Plus kit (Macherey-Nagel). RNA quality and quantity were evaluated  
234 using a BioAnalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies). Stranded  
235 RNA-Seq libraries were constructed from 100 ng high-quality total RNA (RIN > 8)  
236 using the TruSeq Stranded mRNA Library Preparation Kit (Illumina). Paired-end  
237 sequencing of 40 bases length was performed on a NextSeq 500 system (Illumina).

---

238 Pass-filtered reads were mapped using STAR and aligned to mouse reference  
239 genome GRCm38.94 (Chen et al., 2015). Count table of the gene features was  
240 obtained using FeatureCounts (Zhou et al., 2019). Normalization, differential  
241 expression analysis and FPKM (fragments per kilobase of exon per million fragments  
242 mapped) values were computed using EdgeR (Walter et al., 2015). An FPKM filtering  
243 cutoff of 1 in at least one of the 6 samples was applied. A False Discovery Rate  
244 (FDR) of less than or equal to 0.05 was considered significant and a fold change  
245 cutoff of 1.5 was applied to identify differentially expressed genes. Comprehensive  
246 gene list analysis, enriched biological pathways, gene annotation, were based on  
247 Gene Ontology classification system using Metascape (Sajgo et al., 2017). Data  
248 visualization was done using GOplot R package (Livak and Schmittgen, 2001). To  
249 evaluate the expression of the DEGs in RGCs, we used published whole  
250 transcriptome analysis from purified RGCs available on Gene Expression Omnibus  
251 database (GSE87647) (Livak and Schmittgen, 2001).

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

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

---

263 **Statistical analysis**

264 Statistical analysis was performed with GraphPad Prism 8.3.0 (GraphPad Software,  
265 La Jolla California USA). Results are reported as mean  $\pm$  SEM. Nonparametric  
266 Mann-Whitney U test was used to analyze cell counting and qPCR data.  $P$  value  $\leq$   
267 0.05 was considered significant. For OMR assay statistical analysis, a Grubbs test  
268 was performed at 5% to remove outliers followed by 2-way ANOVA (genotype and  
269 spatial frequency) with Bonferroni Post Hoc test.  $P$  value  $\leq$  0.05 was considered  
270 significant.

271

272 **RESULTS**

273 **Retinal progenitor-specific deletion of both *Gsk3 $\alpha$*  and *Gsk3 $\beta$*  results in**  
274 **microphthalmia**

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

284 Hematoxylin and Eosin (H&E) staining revealed major morphological defects  
285 with profound retinal disorganization, including the loss of radial arrangement as well  
286 as folds and aggregates of retinal progenitor cells (RPCs), in *Gsk3 $\alpha^{\text{ff}}$  $\beta^{\text{ff}}$ ; $\alpha$ -Cre* retina  
287 as early as E12.5 (Figure 1B). In addition, blood was detected inside the retinal

---

288 neuroblastic layer. Structure of the retina worsened rapidly during development  
289 although the central part remained unperturbed, consistent with continued *Gsk3*  
290 expression in this region. At and after E14.5, the retina was largely reduced whereas  
291 the eye size itself was comparable to littermate controls (Figure 1B). A large quantity  
292 of blood accumulated inside the eyeball at P2. Finally, growth of the eyeball was  
293 severely reduced leading to microphthalmia in the adult (data not shown).

294

295 **Multiple allelic combinations revealed functional redundancy of *Gsk3α* and**  
296 ***Gsk3β* in retinal development**

297 Severe deleterious effect by the loss of both *Gsk3α* and *Gsk3β* in RPCs in  
298 early development precludes the analysis of late retinal histogenesis. To circumvent  
299 this, we generated animals with different combinations of *Gsk3* deletion (loss of only  
300 one *Gsk3* gene: *Gsk3α<sup>ff</sup>β<sup>+/+</sup>;α-Cre* or *Gsk3α<sup>+/+</sup>β<sup>ff</sup>;α-Cre*, or  $\frac{3}{4}$  deletion: *Gsk3α<sup>ff</sup>β<sup>ff</sup>;α-*  
301 *Cre* or *Gsk3α<sup>ff/+</sup>β<sup>ff</sup>;α-Cre*). Immunoblot analysis using anti-GSK3 antibody  
302 (recognizing both proteins) in 2-month-old animals with different combinations of  
303 *Gsk3α* and *Gsk3β* floxed alleles demonstrated the efficacy of *Gsk3α* and *Gsk3β*  
304 deletion in areas where the Cre recombinase was expressed during early retinal  
305 development (all retinal progenitors at the exception of a stripe located in the dorso-  
306 central region (Roger et al., 2012)) (Figure 2A). IHC analysis using anti-GSK3β  
307 showed ubiquitous expression of *Gsk3β* in adult control retina and its complete loss  
308 in *Gsk3α<sup>ff/+</sup>β<sup>ff</sup>;α-Cre* retina (Figure 2B). At 2-month, retinal histology revealed the  
309 correct laminated architecture with normal photoreceptors and interneurons when  
310 even one allele of *Gsk3α* (Figure 2C, D) or *Gsk3β* (data not shown) was present.  
311 Photopic and scotopic electroretinogram (ERG) recordings, corresponding to cone  
312 and rod function respectively, did not show any significant difference between

---

313 *Gsk3α<sup>f/+</sup>β<sup>ff</sup>;α-Cre* and control retina (Figure 2E, 2F). These results were similar in  
314 mice carrying any combination of *Gsk3* deletion (data not shown). We therefore  
315 conclude that a single allele of wild-type *Gsk3α* or *Gsk3β* is sufficient to rescue  
316 obvious structural and functional defects in the complete absence of GSK3 signaling.

317 **Loss of either *Gsk3α* or *Gsk3β* in RPCs leads to increased number of displaced**  
318 **retinal ganglion cells**

319 Even though a single allele of either *Gsk3α* or *Gsk3β* permitted normal retinal  
320 development (Figure 2), we observed a striking increase in the number of RGCs, as  
321 indicated by Brn3a-positive cells, in the inner nuclear layer (INL) of *Gsk3α<sup>f/+</sup>β<sup>ff</sup>;α-Cre*  
322 retina compared to controls (Figure 3A). Brn3a-positive cells in the INL have been  
323 described as displaced retinal ganglion cells (dRGCs), a rare cell type in the  
324 mammalian retina (Galli-Resta and Ensini, 1996; Young, 1984). All Brn3a-positive  
325 cells in the INL of *Gsk3α<sup>f/+</sup>β<sup>ff</sup>;α-Cre* retina also expressed NF68 that labels cell  
326 bodies and axons of RGCs (Figure 3A). To validate that these Brn3a-positive cells in  
327 the INL were indeed RGCs with axonal projections included in the optic nerve, we  
328 performed retrograde labeling with Rhodamine-Dextran applied onto the optic nerve  
329 of *Gsk3α<sup>f/+</sup>β<sup>ff</sup>;α-Cre* mice. Subsequent 3D reconstructions on flat mount retinas  
330 revealed the presence of numerous fluorescent cell bodies located in the INL  
331 compared to controls (Figure 3B) demonstrating that axons of dRGCs indeed  
332 reached the optic nerve. Thus, Brn3a-positive cells located in the INL of  
333 *Gsk3α<sup>f/+</sup>β<sup>ff</sup>;α-Cre* retinas are indeed RGCs.

334 Increased dRGCs were observed in retinas carrying any combination of *Gsk3*  
335 deletions tested (*Gsk3α<sup>ff</sup>β<sup>+/+</sup>*, *Gsk3α<sup>+/+</sup>β<sup>ff</sup>*, *Gsk3α<sup>f/+</sup>β<sup>ff</sup>* or *Gsk3α<sup>ff</sup>β<sup>f/+</sup>*), with the highest  
336 number detected in *Gsk3α<sup>f/+</sup>β<sup>ff</sup>;α-Cre* mice compared to controls (10-fold increase)  
337 (Figure 3C). Notably, *Gsk3α<sup>f/+</sup>β<sup>f/+</sup>;α-Cre* retina did not display excess of dRGCs (data

---

338 not shown). Interestingly, increase in dRGCs number is not associated with a  
339 significant reduction in the number of RGCs in the GCL, referred to as orthotopic  
340 RGCs (oRGCs) (Figure 3C).

341 Due to their low number in control retina (around 2% of RGCs), dRGCs have  
342 been poorly characterized with very few markers identified, such as Brn3a (Nadal-  
343 Nicolás et al., 2014; Nadal-Nicolás et al., 2012). Immunostaining on sections and flat  
344 mount retinas with additional RGC marker antibodies revealed that dRGCs in  
345 *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ;  $\alpha$ -Cre* retina were also positive for Rbpms (Rodriguez et al., 2014)  
346 confirming their increased number in the INL compared to controls (Figure 3D, E).  
347 Similar results were observed with Islet1 labeling (Figure 3-1A) (Bejarano-Escobar et  
348 al., 2015). Previous work showed that the number of dRGCs varies by retinal domain  
349 (Dräger and Olsen, 1980). Counting of Rbpms- or Brn3a-positive dRGCs on  
350 *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ;  $\alpha$ -Cre* flat mount retinas did not show any significant differences in their  
351 distribution between the dorsal, ventral, nasal and temporal regions (Figure 3-1B).  
352 Finally, Brn3a-positive dRGCs did not express markers of other INL neurons such as  
353 Choline-Acetyltransferase (CHAT, amacrine cells) or Calbindin (horizontal and  
354 amacrine cells) (Figure 3-2). Altogether, our results strongly support the ganglion cell  
355 identity of the displaced Brn3a- and RBPMS-positive cells located in the INL of  
356 *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ;  $\alpha$ -Cre*.

357 To test whether dRGCs in *Gsk3* mutant mice were produced during the same  
358 developmental time window as oRGCs, we performed pulse chase experiments by  
359 injecting EdU at E12.5, at the peak of RGC birth. Retinal sections from one-month-  
360 old animals were then immunolabelled using anti-Brn3a antibody (Figure 4A). In  
361 control and *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ;  $\alpha$ -Cre* retina, we identified 40-50% of RGCs that were  
362 Brn3a/EdU-positive in all layers examined (GCL and INL), indicating that both dRGCs  
363 and oRGCs were born around the same time (Figure 4B). We next examined

---

364 whether dRGCs are usually overproduced during normal retinal development and  
365 eliminated later on. In this context, increased number of dRGCs in *Gsk3 $\alpha$ <sup>ff/+</sup> $\beta$ <sup>ff/+</sup>; $\alpha$ -Cre*  
366 retinas could result from a defect in dRGC riddance occurring during the first two  
367 postnatal weeks, a period of developmental cell death in the retina (Young, 1984). At  
368 P0, the number of Brn3a-positive oRGCs was similar between littermate control and  
369 *Gsk3 $\alpha$ <sup>ff/+</sup> $\beta$ <sup>ff/+</sup>; $\alpha$ -Cre* retina (Figure 4C and D). In contrast, the proportion of Brn3a-  
370 positive cells located in the inner part of neuroblastic layer corresponding presumably  
371 to dRGCs was much lower in control retinas (6 $\pm$ 0.1%) compared to *Gsk3 $\alpha$ <sup>ff/+</sup> $\beta$ <sup>ff/+</sup>; $\alpha$ -Cre*  
372 retinas (30 $\pm$ 1.4%). Thus, dRGCs are not overproduced and eliminated postnatally in  
373 control retina. Our results demonstrate that dRGCs are generated during early waves  
374 of retinogenesis in *Gsk3 $\alpha$ <sup>ff/+</sup> $\beta$ <sup>ff/+</sup>; $\alpha$ -Cre* retina and strongly suggest that GSK3 kinases  
375 play a role in restricting their number during normal retinal development.

376

377 **dRGCs produced in the absence of either *Gsk3 $\alpha$*  or *Gsk3 $\beta$*  project to accessory**  
378 **visual system circuitry**

379 Previous studies in birds and reptiles have reported that dRGCs could be  
380 responsible for optokinetic nystagmus, as they mostly project to the accessory optic  
381 nuclei (AOS) (Cook and Podugolnikova, 2001), which is critical for non-image forming  
382 circuit and image stabilization (Simpson, 1984). To test whether dRGCs in *Gsk3*  
383 mutants project into specific visual nuclei in the brain, including the AOS, we traced  
384 the total pool of RGCs, including dRGCs, with Cholera Toxin beta subunit (CTB). Bi-  
385 lateral injection of CTB, coupled to either an alexa-555 or -647 followed by 3D  
386 imaging, allowed us to trace both ipsi- and contra-lateral projecting axons. We first  
387 confirmed that CTB injections indeed marked the dRGCs based on flat mounts of  
388 retinas after Brn3a and NF68 immunolabelling (Figure 5-1). To visualize the entire

---

389 visual projection network, we carried out whole-brain clearing using iDISCO+  
390 followed by light-sheet fluorescent imaging and 3D reconstruction (Figure 5A).  
391 Complete loss of *Gsk3 $\beta$*  displayed a large increase in ipsilateral projecting RGCs in  
392 one of the three nuclei composing the AOS, the Medial Terminal Nucleus (MTN)  
393 (Simpson, 1984). This terminal nucleus is the main component of the AOS reacting  
394 best to either upward or downward movement and mediates the optokinetic  
395 nystagmus critical for image stabilization (Yonehara et al., 2009). Calculation of the  
396 signal intensity ratio between the ipsilateral and contralateral MTN demonstrated a  
397 significant increase of RGC projections into the ipsilateral MTN in retinas with *Gsk3 $\beta$*   
398 deletion (Figure 5B). This result suggests that excess dRGCs might participate to the  
399 non-image forming circuit.

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

402 We next performed transcriptome analysis using RNA-sequencing in order to  
403 identify molecular changes in adult *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ;  $\alpha$ -Cre* retina and to better characterize  
404 dRGCs. Retinas from *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$*  mice were used as controls. Gene level analysis  
405 revealed 111 differentially expressed genes (DEGs) using filtering criteria of Fold  
406 Change (FC) = 1.5 with a False Discovery rate (FDR) cutoff of  $\leq 0.05$  and a minimum  
407 mean expression value of one FPKM (fragments per kilobase of exon per million  
408 reads mapped) in at least one of the two experimental groups (Figure 6A and Figure  
409 6-1). Pathway analysis of DEGs revealed several statistically significant  
410 overrepresented pathways (Figure 6-2). Biological processes and molecular functions  
411 pathways included 48 DEGs; of these, 33 genes were expressed in RGCs based on  
412 published whole transcriptomic data from purified RGCs (for a total 69 RGC-  
413 expressed genes among the 111 DEGs) (labeled with stars in Figure 8B) (Sajgo et

---

414 al., 2017). Dominance of RGC-expressed genes in our dataset is consistent with the  
415 high number of dRGCs observed in *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* retina.

416 Among interesting candidates dysregulated in the biological processes and molecular  
417 function pathways (Figure 6B and 6-2), we identified *Chrna2*, *Chrna5*, *Chrna7*,  
418 *Chrn4* encoding for postsynaptic subunits of the nicotinic cholinergic receptor. With  
419 the exception of *Chrna2*, all other genes are upregulated in *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* retina.  
420 Most retinal ganglion cells express nicotinic receptors (Kay et al., 2011; Rousso et  
421 al., 2016). Among other potentially relevant genes, the *Grik3* gene product belongs to  
422 the kainate family of glutamate receptors functioning as ligand-activated ion  
423 channels. In direction-selective ganglion cells (DSGCs), glutamate is proposed to be  
424 the main source of excitation (Sweeney et al., 2019, 2014). Finally, *Cartpt*, encoding  
425 for the preprotein CART (Cocaine- And Amphetamine-Regulated Transcript Protein)  
426 that was upregulated in *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* retina was validated by RT-qPCR (Figure  
427 6C). *Cartpt* is specifically expressed in direction-selective RGCs (DS-RGCs) (Rousso  
428 et al., 2016; Sato et al., 2017), suggesting that dRGCs (or at least a subset) in  
429 *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* retina might be DS-RGCs. In support with this hypothesis, we found  
430 some dRGCs in *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* and littermate control retina positive for the  
431 transcription factor *Tbr2*, described as essential for RGC specification participating in  
432 non-image-forming visual circuits (Figure 6D) (Simpson, 1984; Yonehara et al.,  
433 2009). A small subset of dRGCs also expressed *Foxp2*, a transcription factor  
434 involved in DS-RGC differentiation in mice (Figure 6D) (Kim et al., 2009). These two  
435 factors were expressed in a mutually exclusive way in Rpbms-positive dRGCs,  
436 suggesting that dRGCs in *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* might encompass several subtypes.

#### 437 **Optomotor response is impaired in GSK3 mutant**

438 Given that DS-RGCs are reported to drive the optomotor response (OMR) by  
439 projecting mainly into the contralateral AOS (Kim et al., 2009), we tested the OMR of

---

440 *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>; $\alpha$ -Cre* mice. The OMR indices (T<sub>correct</sub> / T<sub>incorrect</sub>) were calculated  
441 from three trials at contrast 1 and 0.5 (Figure 6E). At 100% contrast, the OMR indices  
442 were significantly reduced in *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>; $\alpha$ -Cre* mice compared to controls at 0.05,  
443 0.15 and 0.25 cycles per degree (cpd). The maximum OMR index was observed at  
444 0.15 cpd in controls whereas it reached its maximum at 0.1 in *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>; $\alpha$ -Cre* mice.  
445 At 50% contrast, the OMR indices were also significantly reduced in *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>; $\alpha$ -  
446 Cre* mice compared to controls but to a larger extend between 0.05 and 0.3 cpd. The  
447 maximum OMR index was observed at 0.15 cpd in both controls and *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>; $\alpha$ -  
448 Cre* mice. Altogether, these results demonstrate an impaired OMR in *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>; $\alpha$ -  
449 Cre* mice. These data, together with our transcriptomic and axonal projections  
450 analyses, suggest that at least a subset of dRGCs expressing only one allele of  
451 *Gsk3 $\alpha$*  are DS-RGCs.

452

## 453 DISCUSSION

454 In this study, we report that complete loss of GSK3 in retinal progenitors leads  
455 to microphthalmia in adult mice with severe morphological defects. Such a severe  
456 phenotype was not observed anymore when only one *Gsk3 $\alpha$*  or *Gsk3 $\beta$*  allele was  
457 expressed, confirming the functional redundancy of these two genes. Our results  
458 implicate that the kinase GSK3 as the first reported determinant of dRGCs  
459 determinant during retinal histogenesis. We show that mouse retinas with only one  
460 allele of *Gsk3* exhibit an excessive number of dRGCs. The concomitant large  
461 increase of axonal projections to the ipsilateral MTN, our RNA-Seq data and  
462 optomotor response tests, have led us to propose that these dRGCs are involved in  
463 the detection of image motion direction.

---

464 In pigmented wild-type mouse retina, dRGCs in the INL are a very rare and  
465 poorly-described type of cells, which represent only 2% of RGCs (Li et al., 2012;  
466 Moore et al., 2002). It is therefore striking that dRGC number increases up to 20% of  
467 RGCs when a single copy of *Gsk3 $\alpha$*  is present in retinal progenitors. To our  
468 knowledge, such a high number of dRGCs has never been reported in a  
469 transgenic/mutant animal. A previous study hypothesized that dRGCs are misplaced  
470 in the INL due to an ontogenic aberration rather than representing an independent  
471 class of RGCs (Fite et al., 1981; Krause et al., 2014). Indeed, differential cell  
472 adhesion plays a key role in sorting and migration of retinal cells in their appropriate  
473 layers, especially for RGCs. One can therefore hypothesize that enhanced dRGCs in  
474 mice with a single copy of *Gsk3* is the consequence of increased aberration events.  
475 This hypothesis could be supported by our RNA-Seq data showing the upregulation  
476 of genes coding for collagen subunits (*Col18a1*, *Col4a3*, *Col9a1*, *Col9a2*) and  
477 extracellular matrix proteins in *Gsk3 $\alpha^{f/+}$  $\beta^{ff}$ ; $\alpha$ -Cre* retina, which could favor migration  
478 defects. Noticeably, if it were the case, the increase in dRGCs should be  
479 accompanied by a decrease in oRGCs. However, we found that the number of oRGC  
480 in the GCL is unaltered, strongly suggesting that RGCs in the INL of mice with a  
481 single copy of *Gsk3* represent a specific subtype of RGCs. In support with this,  
482 topographic and quantitative analysis of RGCs in albinos and pigmented rats indicate  
483 that dRGCs are not misplaced by ontogenic mistakes but indeed represent a specific  
484 subpopulation of RGCs (Nadal-Nicolás et al., 2014). GSK3 $\beta$  is involved in neural cell  
485 fate decision by controlling the timing of the activity of bHLH transcription factors,  
486 such as NeuroD or Neurog2 (Karten et al., 1977). If dRGCs are not produced  
487 following ontogenic aberrations but are instead determined by a proper genetic  
488 program, it would be interesting to identify transcription factors involved and seek for  
489 any regulation by GSK3 kinases. Along this line, further studies would allow to better

---

490 understand if the excess of dRGCs is only due to an expanded pool of normally  
491 occurring dRGCs or if their presence is also a consequence of an aberrant migration  
492 during retinal development when GSK3s are not fully active. New sequencing  
493 technology such as single cell RNA-Seq would definitively be an asset to shed more  
494 light on specific markers for dRGCs and to identify key players of dRGC  
495 specification/differentiation. As a distinct cell type, scRNA-seq analysis following high  
496 depth sequencing should highlight a distinct cell cluster in tSNE plots in retina with  
497 only one *Gsk3 $\alpha$*  allele expressed corresponding to dRGCs. The interest and power of  
498 such approach has already been demonstrated for RGC characterization (Rheume  
499 et al., 2018). Whole transcriptome analysis at early time points when RGCs are  
500 produced might also complete such analysis.

501         In reptiles, amphibians and birds, only dRGCs project into the MTN, whereas  
502 in mammals only oRGCs have been reported as projecting into the MTN (Mouritsen  
503 et al., 2004). Our results obtained from anterograde labeling clearly demonstrated a  
504 large increase in ipsilateral MTN projections in absence of *Gsk3 $\beta$* , whereas it was  
505 absent or very dim in control animals. Although this strongly suggests that excess  
506 dRGCs in mutant mice are causing this phenotype, we cannot exclude the possibility  
507 that mutant oRGCs also participate to these ipsilateral MTN projections. However,  
508 contralateral projections did not seem to be affected. Noticeably however, it would be  
509 challenging to observe an increase in dRGC projections into the other areas already  
510 strongly labeled using our anterograde labeling method, especially into the dLGN or  
511 SC. We can speculate that the low number of ipsilateral MTN projections in the  
512 control condition reflects the low number of dRGCs present in the WT retina and  
513 could therefore explain why such result had not been described so far. Altogether,  
514 our results strongly suggest that dRGCs may primarily project into the ipsilateral  
515 MTN. In mice, it has been shown by retrograde labeling from the superior colliculus

---

516 (SC), which receive large amount of RGC projections, that dRGCs/oRGCs project to  
517 one or both SCs (Nießner et al., 2016). Although challenging, similar experiments,  
518 *i.e.* fluorescent dye injection into the ipsilateral MTN, may allow us to discriminate  
519 whether the increased signal in absence of *Gsk3 $\beta$*  originates only from dRGCs and  
520 whether these cells also project into this area in WT retina. In regards to our results, it  
521 is still unclear whether GSK3 function is to limit the number of dRGCs and actively  
522 regulates their correct projection to the contralateral MTN or if GSK3 function is  
523 limited to tightly controlling of dRGC number, which project thereafter to the ipsilateral  
524 MTN on a GSK3-independent manner.

525         Given the very low percentage of dRGCs in the control retina, their function is  
526 poorly studied in mammals. In contrast, dRGC function, projections and topography  
527 have been extensively investigated in bird and reptile retina (Nießner et al., 2016). In  
528 birds, cryptochrome-expressing dRGCs are used as a magnetic compass for  
529 orientation (Pang and Wu, 2011). In European Robin birds, *Erithacus rubecula*, a low  
530 number of dRGCs have been identified but specifically express Cryptochrome 1b  
531 only during nocturnal migration period (Pang and Wu, 2011). In rodents, retrograde  
532 labeling from the optic nerve led to the identification of 16 classes of dRGCs based  
533 on their ramification levels of their dendrites as well as the dendritic field size (Giolli et  
534 al., 2006; Yonehara et al., 2009). Based on dRGC dendrite projections into the IPL, it  
535 has been proposed that most dRGCs in WT retina are functionally more involved in  
536 retinal OFF light pathways (Simpson, 1984; Yonehara et al., 2009). Similar methods  
537 applied to *Gsk3 $\alpha^{f/+}$  $\beta^{f/f}$ ; $\alpha$ -Cre* retina should shed more light on dRGC function and  
538 establish whether all the different classes are present.

539         As part of the AOS, the MTN receives afferent signal from the eye and sends  
540 efferent signal to motor neurons controlling the position of the eye. As such,  
541 optokinetic reflex relies on direction specific retinal projections to the AOS. Neurons

---

542 of the dorsal terminal nucleus (DTN) codes for horizontal stimulus whereas neurons  
543 of the MTN codes for vertical stimulus (Nadal-Nicolás et al., 2014). Therefore, the  
544 direction of image motion relies on DS-RGCs in the retina. The alteration the OMR in  
545 *Gsk3 $\alpha^{fl/+}$  $\beta^{fl/fl}$ ; $\alpha$ -Cre* mice, support the hypothesis that some of the supernumerary  
546 dRGCs are indeed related to motion detection. Although the number of dRGCs was  
547 drastically increased, the OMR was not increased but at the contrary reduced. Such  
548 result might be caused by the higher number of projections to the ipsilateral side  
549 instead of the contralateral one, leading to an alteration of the neuronal circuit  
550 regulating the OMR (Nadal-Nicolás et al., 2014). We also identified in *Gsk3 $\alpha^{fl/+}$  $\beta^{fl/fl}$ ; $\alpha$ -*  
551 *Cre* and control retinas a small subset of dRGCs, which are positive for the  
552 transcription factors Tbr2 and Foxp2, the markers for non-image-forming RGCs and  
553 DS-RGCs respectively (Marquardt et al., 2001). Together with the transcriptomic data  
554 (upregulation of genes such as *Cartpt* expressed in DS-RGCs), these results provide  
555 strong evidence suggesting that the large number of dRGCs in *Gsk3 $\alpha^{fl/+}$  $\beta^{fl/fl}$ ; $\alpha$ -Cre*  
556 retina might indeed be DS-RGCs projecting into the MTN. It has been recently  
557 proposed that dRGCs might be also involved in predator detection by integrating  
558 overhead visual information (Roger et al., 2010). Using suitable and complementary  
559 visual tests, our genetic model could be highly valuable to complete the functional  
560 identification of the dRGCs in visual process.

561 Overall, our results demonstrate a critical role of GSK3 in stringently regulating  
562 the number of a rare type of dRGCs, which has been poorly described as yet. *Gsk3*  
563 mutant mice, with a large number of dRGCs in their retina, offer a unique and  
564 powerful model system to further study the embryonic origin, synaptic connections  
565 and visual function of dRGCs in mammals.

566

---

567 **ACKNOWLEDGEMENT**

568 We are grateful to Dr. Jim Woodgett for providing the *Gsk3* floxed lines and Tudor  
569 Badea and Peter Gruss for the alpha-Cre line. We would also like also to thank  
570 Elodie-Kim Grellier, Yide Mi, and Jessica Gumerson for their help with mouse  
571 colonies and technical support.

572

573 **FUNDING SOURCES**

574 This research was supported by the CNRS, Retina France and by the Intramural  
575 Research Program of the National Eye Institute (EY000450 and EY000546). EK was  
576 supported by the Ernst Ludwig Ehrlich Studienwerk.

577

578 **AUTHOR'S CONTRIBUTION**

579 E.K., R.J.V., C.H., designed and performed the experiments and analyzed the data,  
580 S.L. L.T. and P.S. performed the experiments and analyzed the data, A.C. designed  
581 the experiments, analyzed the data and revised the manuscript, M.P., A.S., J.E.R.  
582 designed the study, analyzed the data, wrote the manuscript with the help of E.K.  
583 R.J.V and C.H. J.E.R supervised the study.

584

585 **REFERENCES**

586 Balkema GW, Dräger UC (1990) Origins of uncrossed retinofugal projections in normal and  
587 hypopigmented mice. *Visual Neuroscience* 4:595–604.  
588 Bejarano-Escobar R, Álvarez-Hernán G, Morona R, González A, Martín-Partido G,  
589 Francisco-Morcillo J (2015) Expression and function of the LIM-homeodomain  
590 transcription factor *Islet-1* in the developing and mature vertebrate retina. *Experimental*  
591 *Eye Research*.  
592 Belle M, Godefroy D, Couly G, Malone SA, Collier F, Giacobini P, Chédotal A (2017)  
593 Tridimensional Visualization and Analysis of Early Human Development. *Cell* 169:161-  
594 173.e12.

- 595 Belle M, Godefroy D, Dominici C, Heitz-Marchaland C, Zelina P, Hellal F, Bradke F,  
596 Chédotal A (2014) A simple method for 3D analysis of immunolabeled axonal tracts in a  
597 transparent nervous system. *Cell reports* 9:1191–201.
- 598 Buhl EH, Dann JF (1988) Morphological diversity of displaced retinal ganglion cells in the  
599 rat: A lucifer yellow study. *Journal of Comparative Neurology* 269:210–218.
- 600 Chen Y, Mccarthy D, Robinson M, Smyth GK (2015) edgeR : differential expression analysis  
601 of digital gene expression data User ' s Guide.
- 602 Cole AR (2012) GSK3 as a Sensor Determining Cell Fate in the Brain. *Frontiers in Molecular*  
603 *Neuroscience* 5:1–10.
- 604 Cook JE, Podugolnikova TA (2001) Evidence for spatial regularity among retinal ganglion  
605 cells that project to the accessory optic system in a frog, a reptile, a bird, and a mammal.  
606 *Visual Neuroscience* 18:289–297.
- 607 Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M,  
608 Gingeras TR (2013) STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* 29:15–  
609 21.
- 610 Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR (2007) Functional redundancy of  
611 GSK-3 $\alpha$  and GSK-3 $\beta$  in Wnt/ $\beta$ -catenin signaling shown by using an allelic  
612 series of embryonic stem cell lines. *Developmental cell* 12:957–71.
- 613 Doble BW, Woodgett JR (2003) GSK-3: Tricks of the trade for a multi-tasking kinase.  
614 *Journal of Cell Science*.
- 615 Doi M, Imatani H, Sasoh M, Uji Y, Yamamura H (1994) Displaced retinal ganglion cells in  
616 the Chinese hamster. *Japanese journal of ophthalmology* 38:139–43.
- 617 Dräger UC, Olsen JF (1980) Origins of crossed and uncrossed retinal projections in  
618 pigmented and albino mice. *Journal of Comparative Neurology* 191:383–412.
- 619 Eom TY, Jope RS (2009) Blocked Inhibitory Serine-Phosphorylation of Glycogen Synthase  
620 Kinase-3 $\alpha/\beta$  Impairs In Vivo Neural Precursor Cell Proliferation. *Biological Psychiatry*  
621 66:494–502.
- 622 Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A (2003) Phosphorylation by glycogen  
623 synthase kinase-3 $\beta$  down-regulates Notch activity, a link for Notch and Wnt pathways.  
624 *Journal of Biological Chemistry* 278:32227–32235.
- 625 Fite K v, Brecha N, Karten HJ, Hunt SP (1981) Displaced ganglion cells and the accessory  
626 optic system of pigeon. *The Journal of comparative neurology* 195:279–88.
- 627 Fu X, Sun H, Klein WH, Mu X (2006) B-Catenin Is Essential for Lamination But Not  
628 Neurogenesis in Mouse Retinal Development. *Developmental Biology* 299:424–437.
- 629 Galli-Resta L, Ensigni M (1996) An intrinsic time limit between genesis and death of  
630 individual neurons in the developing retinal ganglion cell layer. *Journal of Neuroscience*  
631 16:2318–2324.
- 632 Giolli RA, Blanks RHI, Lui F (2006) The accessory optic system: basic organization with an  
633 update on connectivity, neurochemistry, and function. *Progress in brain research*  
634 151:407–40.
- 635 Golpich M, Amini E, Hemmati F, Ibrahim NM, Rahmani B, Mohamed Z, Raymond AA,  
636 Dargahi L, Ghasemi R, Ahmadiani A (2015) Glycogen synthase kinase-3 beta (GSK-3 $\beta$ )  
637 signaling: Implications for Parkinson's disease. *Pharmacological Research*.
- 638 Hamon A, García-García D, Ail D, Bitard J, Chesneau A, Dalkara D, Locker M, Roger JE,  
639 Perron M (2019) Linking YAP to Müller Glia Quiescence Exit in the Degenerative  
640 Retina. *Cell reports* 27:1712-1725.e6.
- 641 Hur E-M, Zhou F-Q (2010) GSK3 signalling in neural development. *Nature reviews*  
642 *Neuroscience* 11:539–551.
- 643 Jacobs KM, Bhawe SR, Ferraro DJ, Jaboin JJ, Hallahan DE, Thotala D (2012) GSK-3 $\beta$ : A  
644 Bifunctional Role in Cell Death Pathways. *International journal of cell biology*  
645 2012:930710.

- 646 Jin YH, Kim H, Oh M, Ki H, Kim K (2009) Regulation of Notch1/NICD and Hes1  
647 expressions by GSK-3 $\alpha/\beta$ . *Molecules and Cells* 27:15–19.
- 648 Karten HJ, Fite K v., Brecha N (1977) Specific projection of displaced retinal ganglion cells  
649 upon the accessory optic system in the pigeon (*Columba livia*). *Proceedings of the*  
650 *National Academy of Sciences of the United States of America* 74:1753–1756.
- 651 Kay JN, de la Huerta I, Kim IJ, Zhang Y, Yamagata M, Chu MW, Meister M, Sanes JR  
652 (2011) Retinal ganglion cells with distinct directional preferences differ in molecular  
653 identity, structure, and central projections. *Journal of Neuroscience* 31:7753–7762.
- 654 Kim W-Y, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR, Snider WD (2009) GSK-3 is a  
655 master regulator of neural progenitor homeostasis. *Nature neuroscience* 12:1390–1397.
- 656 Krause M, Distler C, Hoffmann KP (2014) Retinal ganglion cells projecting to the accessory  
657 optic system in optokinetic blind albinotic rats are direction-selective. *European Journal*  
658 *of Neuroscience* 40:2274–2282.
- 659 Kremer A (2011) GSK3 and Alzheimer’s disease: facts and fiction.... *Frontiers in Molecular*  
660 *Neuroscience* 4:1–10.
- 661 Kretschmer F, Kretschmer V, Kunze VP, Kretzberg J (2013) OMR-arena: Automated  
662 measurement and stimulation system to determine mouse visual thresholds based on  
663 optomotor responses. *PLoS ONE* 8:78058.
- 664 Kretschmer F, Sajgo S, Kretschmer V, Badea TC (2015) A system to measure the Optokinetic  
665 and Optomotor response in mice. *Journal of Neuroscience Methods* 256:91–105.
- 666 Li DW, Liu ZQ, Chen W, Yao M, Li GR (2014) Association of glycogen synthase kinase-3 $\beta$   
667 with Parkinson’s disease (Review). *Molecular Medicine Reports*.
- 668 Li S, Mattar P, Zinyk D, Singh K, Chaturvedi C-P, Kovach C, Dixit R, Kurrasch DM, Ma Y-  
669 C, Chan JA, Wallace V, Dilworth FJ, Brand M, Schuurmans C (2012) GSK3 temporally  
670 regulates neurogenin 2 proneural activity in the neocortex. *The Journal of neuroscience :*  
671 *the official journal of the Society for Neuroscience* 32:7791–805.
- 672 Liao Y, Smyth GK, Shi W (2014) FeatureCounts: An efficient general purpose program for  
673 assigning sequence reads to genomic features. *Bioinformatics* 30:923–930.
- 674 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time  
675 quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods* 25:402–408.
- 676 Marcus EA, Kintner C, Harris W (1998) The role of GSK3 $\beta$  in regulating neuronal  
677 differentiation in *Xenopus laevis*. *Molecular and Cellular Neurosciences* 12:269–280.
- 678 Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P (2001)  
679 Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* 105:43–55.
- 680 Maurer U, Preiss F, Brauns-Schubert P, Schlicher L, Charvet C (2014) GSK-3 - at the  
681 crossroads of cell death and survival. *Journal of Cell Science* 127:1369–1378.
- 682 Medina M, Garrido JJ, Wandosell FG (2011) Modulation of GSK-3 as a Therapeutic Strategy  
683 on Tau Pathologies. *Frontiers in Molecular Neuroscience* 4:1–10.
- 684 Moore KB, Schneider ML, Vetter ML (2002) Posttranslational mechanisms control the timing  
685 of bHLH function and regulate retinal cell fate. *Neuron* 34:183–195.
- 686 Mouritsen H, Janssen-Bienhold U, Liedvogel M, Feenders G, Stalleicken J, Dirks P, Weiler R  
687 (2004) Cryptochromes and neuronal-activity markers colocalize in the retina of  
688 migratory birds during magnetic orientation. *Proceedings of the National Academy of*  
689 *Sciences of the United States of America* 101:14294–9.
- 690 Nadal-Nicolás FM, Jiménez-López M, Salinas-Navarro M, Sobrado-Calvo P, Albuquerque-  
691 Béjar JJ, Vidal-Sanz M, Agudo-Barriuso M (2012) Whole Number, Distribution and Co-  
692 Expression of Brn3 Transcription Factors in Retinal Ganglion Cells of Adult Albino and  
693 Pigmented Rats. *PLoS ONE* 7:e49830.
- 694 Nadal-Nicolás FM, Salinas-Navarro M, Jiménez-López M, Sobrado-Calvo P, Villegas-  
695 Páez MP, Vidal-Sanz M, Agudo-Barriuso M (2014) Displaced retinal ganglion cells  
696 in albino and pigmented rats. *Frontiers in Neuroanatomy* 8:1–21.

- 697 Nießner C, Gross JC, Denzau S, Peichl L, Fleissner G, Wiltschko W, Wiltschko R (2016)  
698 Seasonally changing cryptochrome 1b expression in the retinal ganglion cells of a  
699 migrating passerine bird. *PLoS ONE* 11:e0150377.
- 700 Ouchi Y, Baba Y, Koso H, Taketo MM, Iwamoto T, Aburatani H, Watanabe S (2011)  $\beta$ -  
701 Catenin signaling regulates the timing of cell differentiation in mouse retinal progenitor  
702 cells. *Molecular and Cellular Neuroscience* 46:770–780.
- 703 Pachenari N, Kiani S, Javan M (2017) Inhibition of glycogen synthase kinase 3 increased  
704 subventricular zone stem cells proliferation. *Biomedicine & pharmacotherapy =*  
705 *Biomedecine & pharmacotherapie* 93:1074–1082.
- 706 Pang J-J, Wu SM (2011) Morphology and immunoreactivity of retrogradely double-labeled  
707 ganglion cells in the mouse retina. *Investigative ophthalmology & visual science*  
708 52:4886–96.
- 709 Pérezleón JA, Osorio-Paz I, Francois L, Salceda R (2013) Immunohistochemical localization  
710 of glycogen synthase and GSK3 $\beta$ : Control of glycogen content in retina. *Neurochemical*  
711 *Research* 38:1063–1069.
- 712 Rheaume BA, Jereen A, Bolisetty M, Sajid MS, Yang Y, Renna K, Sun L, Robson P,  
713 Trakhtenberg EF (2018) Single cell transcriptome profiling of retinal ganglion cells  
714 identifies cellular subtypes. *Nature Communications* 9.
- 715 Rodriguez AR, de Sevilla Müller LP, Brecha NC (2014) The RNA binding protein RBPM5 is  
716 a selective marker of ganglion cells in the mammalian retina. *Journal of Comparative*  
717 *Neurology* 522:1411–1443.
- 718 Roger JEJE, Nellissery J, Kim DSDSDS, Swaroop A (2010) Sumoylation of bZIP  
719 transcription factor NRL modulates target gene expression during photoreceptor  
720 differentiation. *Journal of Biological Chemistry* 285:25637–25644.
- 721 Roger JEJEE, Ranganath K, Zhao L, Cojocar RIRIIRI, Brooks M, Gotoh N, Veleri S,  
722 Hiriyanna A, Rachel RARARA, Campos MMMMM, Fariss RNRNN, Wong WTTWT,  
723 Swaroop A (2012) Preservation of cone photoreceptors after a rapid yet transient  
724 degeneration and remodeling in cone-only *Nrl*<sup>-/-</sup> mouse retina. *J Neurosci* 32:528–541.
- 725 Rouso DL, Qiao M, Kagan RD, Yamagata M, Palmiter RD, Sanes JR (2016) Two Pairs of  
726 ON and OFF Retinal Ganglion Cells Are Defined by Intersectional Patterns of  
727 Transcription Factor Expression. *Cell reports* 15:1930–44.
- 728 S H, H W (2000) Immunocytochemical analysis of the mouse retina. *The Journal of*  
729 *comparative neurology* 424:1–23.
- 730 Sajgo S, Ghinia MG, Brooks M, Kretschmer F, Chuang K, Hiriyanna S, Wu Z, Popescu O,  
731 Badea TC (2017) Molecular codes for cell type specification in *Brn3* retinal ganglion  
732 cells. *Proceedings of the National Academy of Sciences of the United States of America*  
733 114:E3974–E3983.
- 734 Sato C, Iwai-Takekoshi L, Ichikawa Y, Kawasaki H (2017) Cell type-specific expression of  
735 FoxP2 in the ferret and mouse retina. *Neuroscience Research* 117:1–13.
- 736 Shimizu T, Kagawa T, Inoue T, Nonaka A, Takada S, Aburatani H, Taga T (2008) Stabilized  
737 beta-catenin functions through TCF/LEF proteins and the Notch/RBP-Jkappa complex to  
738 promote proliferation and suppress differentiation of neural precursor cells. *Molecular*  
739 *and cellular biology* 28:7427–41.
- 740 Simpson JI (1984) The Accessory Optic System. *Annual Review of Neuroscience* 7:13–41.
- 741 Sweeney NT, James KN, Nistorica A, Lorig-Roach RM, Feldheim DA (2019) Expression of  
742 transcription factors divides retinal ganglion cells into distinct classes. *Journal of*  
743 *Comparative Neurology* 527:225–235.
- 744 Sweeney NT, Tierney H, Feldheim DA (2014) *Tbr2* is required to generate a neural circuit  
745 mediating the pupillary light reflex. *The Journal of neuroscience : the official journal of*  
746 *the Society for Neuroscience* 34:5447–53.

- 
- 747 Wall DS, Mears AJ, McNeill B, Mazerolle C, Thurig S, Wang Y, Kageyama R, Wallace VA  
748 (2009) Progenitor cell proliferation in the retina is dependent on Notch-independent  
749 Sonic hedgehog/Hes1 activity. *Journal of Cell Biology* 184:101–112.
- 750 Walter W, Sánchez-Cabo F, Ricote M (2015) GOplot: an R package for visually combining  
751 expression data with functional analysis. *Bioinformatics (Oxford, England)* 31:2912–4.
- 752 Wang B, Li Y (2006) Evidence for the direct involvement of  $\beta$ TrCP in Gli3 protein  
753 processing. *Proceedings of the National Academy of Sciences of the United States of*  
754 *America* 103:33–38.
- 755 Yonehara K, Ishikane H, Sakuta H, Shintani T, Nakamura-Yonehara K, Kamiji NL, Usui S,  
756 Noda M (2009) Identification of retinal ganglion cells and their projections involved in  
757 central transmission of information about upward and downward image motion. *PLoS*  
758 *ONE* 4:e4320.
- 759 Young RW (1984) Cell death during differentiation of the retina in the mouse. *Journal of*  
760 *Comparative Neurology* 229:362–373.
- 761 Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda  
762 SK (2019) Metascape provides a biologist-oriented resource for the analysis of systems-  
763 level datasets. *Nature Communications* 10:1523.
- 764

765

---

766 **FIGURE AND EXTENDED DATA LEGENDS**

767 **Figure 1. Developmental defects and microphthalmia in *Gsk3*-deficient retina**  
768 **with aberrant nuclear translocation of  $\beta$ -catenin, a key effector of the Wnt**  
769 **canonical pathway.** (A) Immunohistochemistry (IHC) of E12.5 retina from *Gsk3 $\alpha$ <sup>ff</sup> $\beta$ <sup>ff</sup>*  
770 mice expressing or not  $\alpha$ -Cre using a pan-GSK3 antibody (green) shows efficient  
771 deletion at the periphery where the Cre expression has been previously reported  
772 (delimited by dashed-line) Scale bar: 100  $\mu$ m. (B) Hematoxylin and eosin (H&E)  
773 staining on methacrylate sections at E12.5, E14.5 and P2 reveals large retinal  
774 morphogenesis defects in *Gsk3 $\alpha$ <sup>ff</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* with blood invasion into the eyeball  
775 (showed by white arrow). L, Lens; NR, neural retina. Scale bar: 100  $\mu$ m at E12.5 and  
776 E14.5. 500  $\mu$ m at P2. For the magnification of P14.5, scale bar: 50  $\mu$ m.

777

778 **Figure 2. One allele of either *Gsk3 $\alpha$*  or *Gsk3 $\beta$*  is sufficient for the development**  
779 **of a functional retina.** (A) Immunoblot analysis of protein extracts from 2-month-old  
780 animals with different combination of *Gsk3 $\alpha$*  and *Gsk3 $\beta$*  floxed alleles (*Gsk3 $\alpha$ <sup>ff</sup> $\beta$ <sup>+/+</sup>,*  
781 *Gsk3 $\alpha$ <sup>+/+</sup> $\beta$ <sup>ff</sup>,* *Gsk3 $\alpha$ <sup>ff/+</sup> $\beta$ <sup>ff</sup>* or *Gsk3 $\alpha$ <sup>ff</sup> $\beta$ <sup>ff/+</sup>*) with or without Cre recombinase using anti-  
782 panGSK3 antibody (recognizing both isoforms) reveals decreased expression of  
783 GSK3 $\alpha$  or GSK3 $\beta$  (arrowheads).  $\alpha$ -Tubulin is used as loading control. (B) IHC on 2-  
784 month-old retinal sections from control and *Gsk3 $\alpha$ <sup>ff/+</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* retinas with or without  
785 Cre recombinase using anti-GSK3 $\beta$  antibody (red) showing ubiquitous *Gsk3 $\beta$*   
786 expression in all retinal layers, whereas its expression is lost in the Cre-expressing  
787 retina. (C) Expression of only one *Gsk3* allele (*Gsk3 $\alpha$* ) is sufficient for proper  
788 photoreceptor development. IHC using anti-Rhodopsin (Rho, red) and anti-Cone  
789 arrestin (Arr3, red) antibodies to label rod and cone photoreceptors, respectively. (D)  
790 Expression of only one *Gsk3* allele (*Gsk3 $\alpha$* ) is sufficient for proper interneuron

791 development. IHC using anti-Calretinin (Calr, green) and anti-Calbindin (Calb, red)  
792 antibodies to label horizontal and amacrine cells, respectively. (B-D) onl, outer  
793 nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 20 $\mu$ m. (E, F)  
794 Electroretinogram (ERG) recording in 2-month-old  $Gsk3\alpha^{f/+}\beta^{f/f};\alpha-Cre$  animals and  
795 littermate controls. Photopic (cones) (E) and scotopic (rods) (F) response in  
796  $Gsk3\alpha^{f/+}\beta^{f/f};\alpha-Cre$  animals are similar to controls. Mean  $\pm$  SEM intensity response  
797 curves of a- and b-wave responses averaged from 8 biological replicates of each  
798 genotype.

799

800 **Figure 3. Gradual loss of  $Gsk3\alpha$  and/or  $Gsk3\beta$  leads to an increased number of**  
801 **Brn3a-positive retinal ganglion cells displaced in the inner nuclear layer (INL)**  
802 **of adult retina.** (A) Brn3a (red) and NF68 (green) IHC on 2-month-old  $Gsk3\alpha^{f/+}\beta^{f/f};\alpha-$   
803  $Cre$  mouse retina reveals the presence of supernumerary displaced retinal ganglion  
804 cells (dRGCs, arrows) in the INL of  $Gsk3\alpha^{f/+}\beta^{f/f};\alpha-Cre$  compared to littermate  
805 controls. Top panel represents control retinas, middle panel a peripheral retinal area,  
806 and bottom panel a more central area. Scale bar: 20  $\mu$ m. (B) dRGCs send their  
807 axons into the optic nerve. Visualization of dRGCs after 3D reconstruction of 2-  
808 month-old flat mounted retina of control and  $Gsk3\alpha^{f/+}\beta^{f/f};\alpha-Cre$  animals following  
809 retrograde labeling with Rhodamin-Dextran applied onto the optic nerve. inl: inner  
810 nuclear layer, gcl: ganglion cell layer. (C) Gradual loss of  $Gsk3\alpha$  and  $Gsk3\beta$  alleles  
811 ( $Gsk3\alpha^{f/f}\beta^{+/+}$ ,  $Gsk3\alpha^{+/+}\beta^{f/f}$ ,  $Gsk3\alpha^{f/+}\beta^{f/f}$  or  $Gsk3\alpha^{f/f}\beta^{f/+}$ ) leads to a gradual increase of  
812 Brn3a-positive RGCs located to the INL, with the highest number observed in  
813  $Gsk3\alpha^{f/+}\beta^{f/f};\alpha-Cre$  animals. Left histograms represent counting of the total number of  
814 Brn3a-positive cells per section located in the GCL (*left panel*) or in the INL (*middle*  
815 *panel*). Right histogram represents the percentage of the dRGCs among the total  
816 number of Brn3a-positive cells per section for each combination. Mean  $\pm$  SEM values

---

817 are presented from 4 biological replicates. A nonparametric Mann-Whitney U test  
818 was applied, \* indicates  $P \leq 0.05$ . (D) Brn3a (red) and Rbpms (green) IHC on 2-  
819 month-old mouse retina reveal the co-expression of these two RGC markers  
820 (dRGCs, arrows) in the INL of both  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre dRGCs and in littermate  
821 controls. Scale bar: 20  $\mu\text{m}$ . (E) Flat mounted retina from  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre and  
822 littermate controls labelled with anti-Rbpms antibody demonstrated the large number  
823 of Rbpms-positive dRGCs in the INL of  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre mice. See Extended Data  
824 Figure 3-1 for Islet1 and Rbpms co-localization used to complete dRGC  
825 characterization and the repartition of the dRGCs in the retina. See Extended Data  
826 Figure 3-2 showing that Brn3a-positive dRGCs are not positive for amacrine or  
827 horizontal cell markers.

828

829 **Figure 4. dRGCs are produced in the same differentiation wave as oRGC**  
830 **located in the GCL.** (A) EdU- (green) and Brn3a-positive cells (red) were found both  
831 in the GCL and in the INL of 30-days old  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre animals after a single  
832 injection of EdU at E12.5. (B) Percentage of EdU- and Brn3a-positive cells located  
833 either in the GCL or in the INL among total number of Brn3a-positive cells. Mean  $\pm$   
834 SEM values are presented from 3-4 biological replicates. A nonparametric Mann-  
835 Whitney U test was applied, ns: not significant (C) Brn3a (red) and NF68 (green)  
836 immunostaining on P0 mouse retina revealed that a large number of dRGCs were  
837 already present in  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre but they were fewer in littermate controls (white  
838 arrows). (D) Left stacked histogram represents counting of the total number of Brn3a-  
839 positive cells per section located in the GCL (white bars) and in the INL (black bars)  
840 of  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre retina. Right histogram represents the percentage of the dRGCs  
841 among the total number of Brn3a-positive cells per section. Mean  $\pm$  SEM values are  
842 presented from 6 biological replicates. A nonparametric Mann-Whitney U test was

843 applied, \*\* indicates  $P \leq 0.01$ . inl, inner nuclear layer; gcl, ganglion cell layer. Scale  
844 bar: 20 $\mu$ m.

845

846 **Figure 5. Lack of Gsk3 $\beta$  results in RGC projections into the ipsilateral Medial**

847 **Terminal Nucleus.** (A) All panels are light sheet fluorescence microscopy of solvent-

848 cleared adult brain from control, *Gsk3 $\alpha^{ff/\beta^{+/+}}$ ;  $\alpha$ -Cre*, *Gsk3 $\alpha^{+/+}\beta^{ff/}$ ;  $\alpha$ -Cre* and

849 *Gsk3 $\alpha^{ff/\beta^{ff/}}$ ;  $\alpha$ -Cre* animals after intravitreal injection of CTB coupled to either an

850 Alexa-555 or -647. Ipsilateral projections of RGCs into the MTN was observed in the

851 absence of *Gsk3 $\beta$*  expression. SC, superior colliculus; NOT, nucleus of optic tract;

852 dLGN, dorsal lateral geniculate nucleus; vLGN, ventral lateral geniculate nucleus;

853 IGL, intergeniculate leaflet; OPT, Olivary Pretectal Nucleus; dMTN, dorsal medial

854 terminal nucleus; MTN, medial terminal nucleus; vMTN, ventral medial terminal

855 nucleus; OT, Optic tract; SCN, suprachiasmatic nucleus; ON, optic nerve. Scale bar:

856 1mm; \* indicates the ipsilateral MTN. (B) Quantification of the signal intensity ratio

857 between ipsilateral and contralateral MTN in controls and *Gsk3* mutants (including

858 *Gsk3 $\alpha^{ff/\beta^{+/+}}$ ;  $\alpha$ -Cre*, *Gsk3 $\alpha^{+/+}\beta^{ff/}$ ;  $\alpha$ -Cre*, and *Gsk3 $\alpha^{ff/\beta^{ff/}}$ ;  $\alpha$ -Cre*). A nonparametric

859 Mann-Whitney U test was applied, ns: non-significant, \*\*  $P \leq 0.01$ . See Extended

860 Data Figure 5-1 for co-staining of the CTB-positive cells with Brn3a and NF68.

861

862 **Figure 6. Whole transcriptome meta-analysis suggests that dRGC in**

863 ***Gsk3 $\alpha^{ff/\beta^{ff/}}$ ;  $\alpha$ -Cre* retina are direction-selective ganglion cells.** (A) Volcano plot

864 representation of differentially expressed genes between *Gsk3 $\alpha^{ff/\beta^{ff/}}$ ;  $\alpha$ -Cre* and

865 control retina plotted on the x-axis (log<sub>2</sub> scale). FDR adjusted significance is plotted

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

867 genes in *Gsk3 $\alpha^{ff/\beta^{ff/}}$ ;  $\alpha$ -Cre* retina, respectively. Vertical dashed lines represent

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

---

869 of DEGs related to GO annotations belonging to either molecular functions (MF) or  
870 biological process (BP). Overlaps in GO annotation amongst genes within each  
871 category are visualized. \* correspond to genes expressed in previously published  
872 purified RGCs (blue, slightly expressed genes in RGCs between 1 and 5 FPKM; red,  
873 highly expressed genes in RGCs more than 5 FPKM). (C) RT-qPCR validation of  
874 selected DEGs identified by RNA-seq analysis. Differential expression analysis by  
875 RT-qPCR of *Cartpt*, *Th*, *Epha2*, *Cplx1*, *Chrna5*, *Chrna2*, *Chrna7*, *Chrn4* in  
876 *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* retina at 2-months of age, relative to littermate control retina levels.  
877 All values are expressed as the Mean  $\pm$  SEM from three biological replicates. A  
878 nonparametric Mann-Whitney U test was applied, \* indicates  $P \leq 0.05$ . (D) IHC on 2-  
879 month-old mouse retina reveals the presence of a subset of dRGCs (Rbpms-positive  
880 dRGCs, red) in *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* expressing either the transcription factor *Tbr2*  
881 (grey) or *Foxp2* (green). Arrows indicate *Tbr2* and Rbpms-positive dRGCs;  
882 arrowheads represent *Foxp2* and Rbpms-positive dRGCs. onl, outer nuclear layer;  
883 inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 50  $\mu$ m. (E) The mean OMR  
884 indices ( $\pm$ SEM) are plotted as a function of spatial frequency for each genotype  
885 ( $n=13$  for *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup> and 18 for *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* genotype). The baseline (1; dashed  
886 line) represents unspecific head movements and no response to the stimulus. OMR  
887 at 100% and 50% contrast in *Gsk3 $\alpha$ <sup>f/+</sup> $\beta$ <sup>ff</sup>;  $\alpha$ -Cre* mice (dashed line) and controls  
888 (black line). A Grubbs test was performed at 5% to remove outliers followed by 2-way  
889 ANOVA, \* indicates  $P \leq 0.05$ , \*\* indicates  $P \leq 0.01$ , \*\*\* indicates  $P \leq 0.001$ . See  
890 Extended Data Figure 6-1 for the hierarchical clustering of the DEGs. See Extended  
891 Data Figure 6-2 for pathway analysis results.*

892

893 **EXTENDED DATA**

---

894 **Figure 3-1. dRGCs express the nuclear factor Islet-1.** (A) IHC on 2-month-old  
895 mouse retina reveals that most dRGCs (Rbpms-positive dRGCs, white arrows, red)  
896 in the INL of  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre and littermate controls were positive for Islet-1  
897 (green), a marker expressed in the nuclei of ganglion cells, and of cholinergic  
898 amacrine cells, ON-bipolar cells, and subpopulations of horizontal cells. onl, outer  
899 nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 50  $\mu$ m. (B)  
900 Counting on flat mount of Rbpms- or Brn3a- positive cells located in the INL at the  
901 dorsal, ventral, nasal and temporal part of control and  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre retina.  
902 Histogram represents the number of Brn3a- or Rbpms-positive cells per field. Mean  $\pm$   
903 SEM values are presented from 4 biological replicates.

904

905 **Figure 3-2. Brn3a-positive cells located in the INL of  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre retina**  
906 **are dRGCs.** Brn3a-positive RGCs located in the INL of  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre retina do  
907 not express markers of other INL neurons such as Choline-Acetyltransferase (Chat)  
908 or Calbindin (Calb). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell  
909 layer. Arrowheads indicates Brn3a-positive dRGCs. Scale bar: 20 $\mu$ m.

910

911 **Figure 5-1. Intravitreal injection of CTB labels dRGCs.** After intravitreal injection of  
912 CTB coupled to an Alexa-555 (red) in  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre eye led to the labelling of  
913 Brn3a- (green) and NF68-positive (grey) cells located in the INL. Scale bars: 20 $\mu$ m

914

915 **Figure 6-1. Hierarchical clustering of the identified differentially expressed**  
916 **genes.** Hierarchical clustering representing the 111 DEGs ( $\text{abs(FC)}\geq 1.5$ ;  $\text{FDR}\leq 0.05$ ;  
917  $\text{FPKM}>1$ ) between 2-month-old  $Gsk3\alpha^{f/+}\beta^{ff/}$ ;  $\alpha$ -Cre retina and littermate control were  
918 clustered by their Z-score. Each column for each genotype corresponds to one

---

919 sample. For both groups, triplicates were analyzed. Left panel corresponds to the  
920 downregulated genes; Right panel corresponds to the upregulated genes.

921

922 **Figure 6-2. Identification of enriched pathways from DEGs identified in 2-**

923 **month-old  $Gsk3\alpha^{f/+}\beta^{ff}$ ;  $\alpha$ -Cre retina.** (A) Gene ontology (GO) annotations of DEGs

924 in  $Gsk3\alpha^{f/+}\beta^{ff}$ ;  $\alpha$ -Cre retina compared to littermate controls. Top over-represented

925 pathways for Biological process (BP), Molecular Function (MF), KEGG (Kyoto

926 Encyclopedia of Genes and Genomes) and TRRUST (Transcriptional Regulatory

927 Relationships Unrevealed by Sentence-based Text mining) were identified by

928 enrichment analysis using Metascape. (B) Circular visualization for BP and MF of GO

929 enrichment analysis. Down-regulated genes (blue dots) and up-regulated genes (red

930 dots) within each GO pathway are plotted based on logFC. Z-score bars indicate if an

931 entire GO category is more likely to be increased or decreased based on the genes

932 within it.

933

934

935

936

937

938

939

940

941

942

943

944

945

946

947

948

949

950 **Table 1. List of primary and secondary antibodies used for**  
 951 **immunohistochemistry (IF) and western blot (WB)**

952 **Primary antibodies**

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION (IF)	DILUTION (WB)
$\alpha$ -tubulin	mouse	SIGMA	T5168		1:200.000
GSK3 $\alpha/\beta$	mouse	Thermo Scientific	Fisher 44-610	1:250	1:1000
GSK3 $\beta$	mouse	BD	610201	1:250	
Brn3a	mouse	Santa Cruz	sc-8429	1:200	
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	
Chat	Goat	Millipore	AB144P	1 :100	

953

954 **Secondary antibodies**

ANTIGENE	HOST	SUPPLIER	REFERENCE	DILUTION
Alexa Fluor 555 anti-mouse IgG2A	goat	Thermo Scientific	Fisher A21127	1:1000
Alexa Fluor 555	goat	Thermo	Fisher A21147	1:1000

---

anti-mouse IgG2B		Scientific		
Alexa Fluor 488	donkey	Thermo Fisher	A21206	1:1000
anti-rabbit		Scientific		
Alexa Fluor 488	goat	Thermo Fisher	A21240	1:1000
anti-mouse IgG1		Scientific		
Alexa Fluor 488	goat	Thermo Fisher	A21244	1:1000
anti-rabbit		Scientific		
HRP anti-mouse	goat	Sigma-Aldrich	A4416	1:5000
IgG				

Gene name	Primer F	Primer R
<i>Cartpt</i>	5'-TAAAGTTTTCGTTCCCCTCAG-3'	5'-CAACACCATTTCGAGGCATTCT-3'
<i>Th</i>	5'-ACTATGCCTCTCGTATCCAGC-3'	5'-CGGATGGTGTGAGGACTGTC-3'
<i>Epha2</i>	5'-GACCTCCCCATCTTCATTTGG-3'	5'-GCGTACAGTGCCCTAGTCATA-3'
<i>Cplx1</i>	5'-GGTGATGAGGAAAAGGACCCC-3'	5'-TCTTGGCGTACTTTGCTTTGC-3'
<i>Chrna5</i>	5'-CTTGAGTACCAACACTGTCCG-3'	5'-CCAGTACTCCAAAGATGCCCT-3'
<i>Chrna2</i>	5'-CATTATCGTCTGCTTCCTGGG-3'	5'-CTTGGAGCCAACATGAGGGA-3'
<i>Chrna7</i>	5'-CTGTAGCTGTCCGTCTTGAGA-3'	5'-CAATGATATGCCGGTGATGGG-3'
<i>Chrnb4</i>	5'-AAACTGATCTGGCTACCTCCC-3'	5'-GTAGAGAGTCCAGGAGATGCC-3'

Alexa Fluor 488	donkey	Thermo Fisher	A11055	1:1000
anti-goat		Scientific		

955

956

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











