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

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ABSTRACT

Glycogen Synthase Kinase 3 (GSK) proteins (GSK3α and GSK3β) are key mediators of signaling pathways, with crucial roles in coordinating fundamental biological processes during neural development. Here we show that the complete loss of GSK3 signaling in mouse retinal progenitors leads to microphthalmia with broad morphological defects. A single wild-type allele of either *Gsk3α* or *Gsk3β* is able to rescue this phenotype. In this genetic context, all cell types are present with a functional retina. However, we unexpectedly detect a large number of cells in the inner nuclear layer expressing retinal ganglion cell (RGC)-specific markers (called displaced RGCs, dRGCs) when at least one allele of *Gsk3α* is expressed. Excess dRGCs lead to increased number of axons projecting into the ipsilateral medial terminal nucleus, an area of the brain belonging to the non-image-forming visual circuit and poorly targeted by RGCs in wild-type retina. Transcriptome analysis and optomotor response assay suggest that at least a subset of dRGCs in *Gsk3* mutant mice are direction-selective RGCs. Our study thus uncovers a unique role of GSK3 in controlling the production of ganglion cells in the inner nuclear layer, which correspond to dRGCs, a rare and poorly characterized retinal cell type.

Significance Statement

Glycogen Synthase Kinase 3 (GSK) proteins (Gsk3α or Gsk3β) are key mediators of signaling pathways, especially in the central nervous system but poorly described in the retina. Here we show that the complete loss of GSK3 in mouse retinal progenitors leads to microphthalmia. However, when only one allele of *Gsk3α* or *Gsk3β* are present, all cell types are present with a functional retina. More importantly, we unexpectedly uncover a unique role of GSK3 in controlling the genesis of retinal ganglion cells in the inner nuclear layer which could correspond to a rare and poorly characterized retinal cell type. Therefore, our mouse models potentially offer a unique and powerful model system to study the visual function of dRGCs in mammals.

INTRODUCTION

Glycogen Synthase Kinase 3 alpha (GSK3α) and beta (GSK3β) are functionally redundant serine/threonine kinases encoded by two different genes, sharing 95% identity in their kinase domain (Doble et al., 2007). GSK3 exists at the crossroads of multiple signaling pathways and acts as a key molecular switch to mediate their output and guide distinct cellular processes (Cole, 2012; Doble and Woodgett, 2003; Espinosa et al., 2003; Jin et al., 2009; Shimizu et al., 2008; Wang and Li, 2006). Among the signaling pathways regulated by GSK3 kinases, Wnt canonical pathway is the most well described, with GSK3β inhibition triggering an increase in β-catenin protein levels and its nuclear translocation to activate target gene expression (Doble and Woodgett, 2003).

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

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

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

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

MATERIALS AND METHODS

Animals and tissue collection

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

the National Institutes of Health (ASP#650). *Gsk3α* and *Gsk3β* floxed mice were generously provided by Dr. Jim Woodgett (University of Toronto, Canada). Floxed *Gsk3* mice were mated with those carrying the retina-specific regulatory element of murine *Pax6* driving the expression of the Cre recombinase (*α-Cre*) in retinal progenitors as early as E10.5 generously provided by Peter Gruss (Roger et al., 2012). Mice are on a mixed background C57Bl6/J and 129/SvJ. Animals from either sex were used for experimental procedures. All mouse genotyping was performed as described (Hamon et al., 2019).

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

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

EdU labeling and TUNEL assay

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

Immunoblotting

Frozen retinas were lysed by sonication in lysis buffer (20 mM Na_2HPO4, 250 mM NaCl, 30 mM NaPPi, 0.1% NP40, 5mM EDTA, 5mM DTT) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysates concentration was determined using a Lowry protein assay kit (Bio-Rad) following sonication and centrifugation. Protein supernatants were separated under denaturing condition by SDS-PAGE, transferred onto nitrocellulose membrane and probed with antibodies (listed in Table 1), as described (Belle et al., 2017, 2014). Proteins were visualized using enhanced chemiluminescence kit (Bio-Rad). α-tubulin was used for normalization. Quantification was performed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain at NIH).

Retinal flat mount

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

Electroretinography

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

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

Optomotor response (OMR)

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

OMRs were recorded using two different Michelson contrasts and different spatial frequencies (presented in random order) in the two mouse groups: 100% contrast or 50% contrast (n=13 for *Gsk3α*$^{f/+}$*β*$^{f/f}$ and 18 for *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* genotype). All stimuli were presented for 60 s randomly in either clockwise or counterclockwise direction. The measurements were completed in three trials for each animal. At 100% and 50% contrast, OMRs were recorded in response to sinusoidal gratings at 12 spatial frequencies between 0.0125 and 0.5 cycles per degree (cpd). The number of head

movements recorded at a speed range from 2 to 14 degrees per second in the same direction as the stimulus (T_Correct) and in the opposite direction (T_Incorrect) were used to calculate the OMR indices (T_Correct / T_Incorrect) at each spatial frequency.

Retrograde labeling of retinal ganglion cells

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

Anterograde labeling of retinal ganglion cell projections

Anterograde labeling

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

Tissue Clearing and 3D imaging

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

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

3D imaging/Image acquisition for all samples was performed as previously described (Liao et al., 2014). Acquisitions were done using an ultramicroscope I (LaVision Biotec) with the ImspectorPro software (LaVision Biotec). The step size between each image was fixed at 2 µm with a numerical aperture of 0.120 and 150ms acquisition using a PCO Edge SCMOS CCD camera (2,560 x 2,160 pixel size, LaVision BioTec).

Image analysis

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

Whole transcriptome sequencing and data analysis

Whole transcriptome analysis was performed on three independent biological replicates from *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* and *Gsk3α$^{f/+}$β$^{f/f}$* retina at P60. After harvesting, both retinas for each animal were immediately frozen. RNA was extracted using Nucleospin RNA Plus kit (Macherey-Nagel). RNA quality and quantity were evaluated using a BioAnalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies). Stranded RNA-Seq libraries were constructed from 100 ng high-quality total RNA (RIN > 8) using the TruSeq Stranded mRNA Library Preparation Kit (Illumina). Paired-end sequencing of 40 bases length was performed on a NextSeq 500 system (Illumina).

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

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

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

Statistical analysis

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

RESULTS

Retinal progenitor-specific deletion of both *Gsk3α* and *Gsk3β* results in microphthalmia

We crossed the floxed *Gsk3α*$^{f/f}$*β*$^{f/f}$ mice with *α-Cre* (*αPax6-Cre*) line to generate *Gsk3α*$^{f/f}$*β*$^{f/f}$*;α-Cre* mice in which *Gsk*3 deletion occurs only in retinal progenitors as early as E10.5 (Marquardt et al., 2001). We first validated our model by assessing the efficacy of *Gsk3α* and *Gsk3β* deletion at E12.5 (Figure 1A). Immunohistochemistry (IHC) using an antibody recognizing both GSK3 proteins showed ubiquitous expression in control retinas (Figure 1A). Both *Gsk3* genes were efficiently deleted in the peripheral retina of *Gsk3α*$^{f/f}$*β*$^{f/f}$*;α-Cre* mice, but their expression in the central retina remained preserved consistent with the previously described *α-Cre* expression pattern (Marquardt et al., 2001).

Hematoxylin and Eosin (H&E) staining revealed major morphological defects with profound retinal disorganization, including the loss of radial arrangement as well as folds and aggregates of retinal progenitor cells (RPCs), in *Gsk3α*$^{f/f}$*β*$^{f/f}$*;α-Cre* retina as early as E12.5 (Figure 1B). In addition, blood was detected inside the retinal

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

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

Severe deleterious effect by the loss of both *Gsk3α and Gsk3β* in RPCs in early development precludes the analysis of late retinal histogenesis. To circumvent this, we generated animals with different combinations of *Gsk3* deletion (loss of only one *Gsk3* gene: $Gsk3\alpha^{f/f}\beta^{+/+};\alpha$-*Cre* or $Gsk3\alpha^{+/+}\beta^{f/f};\alpha$-*Cre*, or ¾ deletion: $Gsk3\alpha^{f/f}\beta^{f/+};\alpha$-*Cre* or $Gsk3\alpha^{f/+}\beta^{f/f};\alpha$-*Cre*). Immunoblot analysis using anti-GSK3 antibody (recognizing both proteins) in 2-month-old animals with different combinations of *Gsk3α* and *Gsk3β* floxed alleles demonstrated the efficacy of *Gsk3α* and *Gsk3β* deletion in areas where the Cre recombinase was expressed during early retinal development (all retinal progenitors at the exception of a stripe located in the dorso-central region (Roger et al., 2012)) (Figure 2A). IHC analysis using anti-GSK3β showed ubiquitous expression of *Gsk3β* in adult control retina and its complete loss in $Gsk3\alpha^{f/+}\beta^{f/f};\alpha$-*Cre* retina (Figure 2B). At 2-month, retinal histology revealed the correct laminated architecture with normal photoreceptors and interneurons when even one allele of *Gsk3α* (Figure 2C, D) or *Gsk3β* (data not shown) was present. Photopic and scotopic electroretinogram (ERG) recordings, corresponding to cone and rod function respectively, did not show any significant difference between

313 *Gsk3α*$^{f/+}$*β*$^{f/f}$*;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*$^{f/+}$*β*$^{f/f}$*;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*$^{f/+}$*β*$^{f/f}$*;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*$^{f/+}$*β*$^{f/f}$*;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*$^{f/+}$*β*$^{f/f}$*;α-Cre* retinas are indeed RGCs.

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

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

Due to their low number in control retina (around 2% of RGCs), dRGCs have been poorly characterized with very few markers identified, such as Brn3a (Nadal-NicolÃ¡s et al., 2014; Nadal-Nicolás et al., 2012). Immunostaining on sections and flat mount retinas with additional RGC marker antibodies revealed that dRGCs in *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* retina were also positive for Rbpms (Rodriguez et al., 2014) confirming their increased number in the INL compared to controls (Figure 3D, E). Similar results were observed with Islet1 labeling (Figure 3-1A) (Bejarano-Escobar et al., 2015). Previous work showed that the number of dRGCs varies by retinal domain (Dräger and Olsen, 1980). Counting of Rbpms- or Brn3a-positive dRGCs on *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* flat mount retinas did not show any significant differences in their distribution between the dorsal, ventral, nasal and temporal regions (Figure 3-1B). Finally, Brn3a-positive dRGCs did not express markers of other INL neurons such as Choline-Acetyltransferase (CHAT, amacrine cells) or Calbindin (horizontal and amacrin cells) (Figure 3-2). Altogether, our results strongly support the ganglion cell identity of the displaced Brn3a- and RPBMS-positive cells located in the INL of *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre.*

To test whether dRGCs in *Gsk3* mutant mice were produced during the same developmental time window as oRGCs, we performed pulse chase experiments by injecting EdU at E12.5, at the peak of RGC birth. Retinal sections from one-month-old animals were then immunolabelled using anti-Brn3a antibody (Figure 4A). In control and *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* retina, we identified 40-50% of RGCs that were Brn3a/EdU-positive in all layers examined (GCL and INL), indicating that both dRGCs and oRGCs were born around the same time (Figure 4B). We next examined

whether dRGCs are usually overproduced during normal retinal development and eliminated later on. In this context, increased number of dRGCs in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retinas could result from a defect in dRGC riddance occurring during the first two postnatal weeks, a period of developmental cell death in the retina (Young, 1984). At P0, the number of Brn3a-positive oRGCs was similar between littermate control and *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina (Figure 4C and D). In contrast, the proportion of Brn3a-positive cells located in the inner part of neuroblastic layer corresponding presumably to dRGCs was much lower in control retinas (6±0.1%) compared to *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retinas (30±1.4%). Thus, dRGCs are not overproduced and eliminated postnatally in control retina. Our results demonstrate that dRGCs are generated during early waves of retinogenesis in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina and strongly suggest that GSK3 kinases play a role in restricting their number during normal retinal development.

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

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

visual projection network, we carried out whole-brain clearing using iDISCO+ followed by light-sheet fluorescent imaging and 3D reconstruction (Figure 5A). Complete loss of *Gsk3β* displayed a large increase in ipsilateral projecting RGCs in one of the three nuclei composing the AOS, the Medial Terminal Nucleus (MTN) (Simpson, 1984). This terminal nucleus is the main component of the AOS reacting best to either upward or downward movement and mediates the optokinetic nystagmus critical for image stabilization (Yonehara et al., 2009). Calculation of the signal intensity ratio between the ipsilateral and contralateral MTN demonstrated a significant increase of RGC projections into the ipsilateral MTN in retinas with *Gsk3β* deletion (Figure 5B). This result suggests that excess dRGCs might participate to the non-image forming circuit.

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

We next performed transcriptome analysis using RNA-sequencing in order to identify molecular changes in adult *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina and to better characterize dRGCs. Retinas from *Gsk3α*$^{f/+}$*β*$^{f/f}$ mice were used as controls. Gene level analysis revealed 111 differentially expressed genes (DEGs) using filtering criteria of Fold Change (FC) = 1.5 with a False Discovery rate (FDR) cutoff of ≤ 0.05 and a minimum mean expression value of one FPKM (fragments per kilobase of exon per million reads mapped) in at least one of the two experimental groups (Figure 6A and Figure 6-1). Pathway analysis of DEGs revealed several statistically significant overrepresented pathways (Figure 6-2). Biological processes and molecular functions pathways included 48 DEGs; of these, 33 genes were expressed in RGCs based on published whole transcriptomic data from purified RGCs (for a total 69 RGC-expressed genes among the 111 DEGs) (labeled with stars in Figure 8B) (Sajgo et

al., 2017). Dominance of RGC-expressed genes in our dataset is consistent with the high number of dRGCs observed in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina.

Among interesting candidates dysregulated in the biological processes and molecular function pathways (Figure 6B and 6-2), we identified *Chrna2*, *Chrna5, Chrna7, Chrnb4* encoding for postsynaptic subunits of the nicotinic cholinergic receptor. With the exception of *Chrna2*, all other genes are upregulated in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina. Most retinal ganglion cells express nicotinic receptors (Kay et al., 2011; Rousso et al., 2016). Among other potentially relevant genes, the *Grik3* gene product belongs to the kainate family of glutamate receptors functioning as ligand-activated ion channels. In direction-selective ganglion cells (DSGCs), glutamate is proposed to be the main source of excitation (Sweeney et al., 2019, 2014). Finally, *Cartpt,* encoding for the preprotein CART (Cocaine- And Amphetamine-Regulated Transcript Protein) that was upregulated in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina was validated by RT-qPCR (Figure 6C). *Cartpt* is specifically expressed in direction-selective RGCs (DS-RGCs) (Rousso et al., 2016; Sato et al., 2017), suggesting that dRGCs (or at least a subset) in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* retina might be DS-RGCs. In support with this hypothesis, we found some dRGCs in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* and littermate control retina positive for the transcription factor Tbr2, described as essential for RGC specification participating in non-image-forming visual circuits (Figure 6D) (Simpson, 1984; Yonehara et al., 2009). A small subset of dRGCs also expressed Foxp2, a transcription factor involved in DS-RGC differentiation in mice (Figure 6D) (Kim et al., 2009). These two factors were expressed in a mutually exclusive way in Rpbms-positive dRGCs, suggesting that dRGCs in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* might encompass several subtypes.

Optomotor response is impaired in GSK3 mutant

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

Gsk3α$^{f/+}$*β*$^{f/f}$;*α-Cre* mice. The OMR indices (T_correct / T_incorrect) were calculated from three trials at contrast 1 and 0.5 (Figure 6E). At 100% contrast, the OMR indices were significantly reduced in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* mice compared to controls at 0.05, 0.15 and 0.25 cycles per degree (cpd). The maximum OMR index was observed at 0.15 cpd in controls whereas it reached its maximum at 0.1 in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* mice. At 50% contrast, the OMR indices were also significantly reduced in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* mice compared to controls but to a larger extend between 0.05 and 0.3 cpd. The maximum OMR index was observed at 0.15 cpd in both controls and *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* mice. Altogether, these results demonstrate an impaired OMR in *Gsk3α*$^{f/+}$*β*$^{f/f}$;*α-Cre* mice. These data, together with our transcriptomic and axonal projections analyses, suggest that at least a subset of dRGCs expressing only one allele of *Gsk3*α are DS-RGCs.

DISCUSSION

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

In pigmented wild-type mouse retina, dRGCs in the INL are a very rare and poorly-described type of cells, which represent only 2% of RGCs (Li et al., 2012; Moore et al., 2002). It is therefore striking that dRGC number increases up to 20% of RGCs when a single copy of *Gsk3*α is present in retinal progenitors. To our knowledge, such a high number of dRGCs has never been reported in a transgenic/mutant animal. A previous study hypothesized that dRGCs are misplaced in the INL due to an ontogenic aberration rather than representing an independent class of RGCs (Fite et al., 1981; Krause et al., 2014). Indeed, differential cell adhesion plays a key role in sorting and migration of retinal cells in their appropriate layers, especially for RGCs. One can therefore hypothesize that enhanced dRGCs in mice with a single copy of *Gsk3* is the consequence of increased aberration events. This hypothesis could be supported by our RNA-Seq data showing the upregulation of genes coding for collagen subunits (*Col18a1*, *Col4a3*, *Col9a1*, *Col9a2*) and extracellular matrix proteins in *Gsk3*$\alpha^{f/+}\beta^{f/f}$;*α-Cre* retina, which could favor migration defects. Noticeably, if it were the case, the increase in dRGCs should be accompanied by a decrease in oRGCs. However, we found that the number of oRGC in the GCL is unaltered, strongly suggesting that RGCs in the INL of mice with a single copy of *Gsk3* represent a specific subtype of RGCs. In support with this, topographic and quantitative analysis of RGCs in albinos and pigmented rats indicate that dRGCs are not misplaced by ontogenic mistakes but indeed represent a specific subpopulation of RGCs (Nadal-NicolÃ¡s et al., 2014). GSK3β is involved in neural cell fate decision by controlling the timing of the activity of bHLH transcription factors, such as NeuroD or Neurog2 (Karten et al., 1977). If dRGCs are not produced following ontogenic aberrations but are instead determined by a proper genetic program, it would be interesting to identify transcription factors involved and seek for any regulation by GSK3 kinases. Along this line, further studies would allow to better

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

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

(SC), which receive large amount of RGC projections, that dRGCs/oRGCs project to one or both SCs (Nießner et al., 2016). Although challenging, similar experiments, *i.e.* fluorescent dye injection into the ipsilateral MTN, may allow us to discriminate whether the increased signal in absence of *Gsk3β* originates only from dRGCs and whether these cells also project into this area in WT retina. In regards to our results, it is still unclear whether GSK3 function is to limit the number of dRGCs and actively regulates their correct projection to the contralateral MTN or if GSK3 function is limited to tightly controlling of dRGC number, which project thereafter to the ipsilateral MTN on a GSK3-independent manner.

Given the very low percentage of dRGCs in the control retina, their function is poorly studied in mammals. In contrast, dRGC function, projections and topography have been extensively investigated in bird and reptile retina (Nießner et al., 2016). In birds, cryptochrome-expressing dRGCs are used as a magnetic compass for orientation (Pang and Wu, 2011). In European Robin birds, *Erithacus rubecula,* a low number of dRGCs have been identified but specifically express Cryptochrome 1b only during nocturnal migration period (Pang and Wu, 2011). In rodents, retrograde labeling from the optic nerve led to the identification of 16 classes of dRGCs based on their ramification levels of their dendrites as well as the dendritic field size (Giolli et al., 2006; Yonehara et al., 2009). Based on dRGC dendrite projections into the IPL, it has been proposed that most dRGCs in WT retina are functionally more involved in retinal OFF light pathways (Simpson, 1984; Yonehara et al., 2009). Similar methods applied to *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* retina should shed more light on dRGC function and establish whether all the different classes are present.

As part of the AOS, the MTN receives afferent signal from the eye and sends efferent signal to motor neurons controlling the position of the eye. As such, 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-NicolÃ¡s et al., 2014). Therefore, the
544 direction of image motion relies on DS-RGCs in the retina. The alteration the OMR in
545 *Gsk3α$^{f/+}$β$^{f/f}$;α-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-NicolÃ¡s et al., 2014). We also identified in *Gsk3α$^{f/+}$β$^{f/f}$;α-*
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 *Gsk3α$^{f/+}$β$^{f/f}$;α-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

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AUTHOR'S CONTRIBUTION

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

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FIGURE AND EXTENDED DATA LEGENDS

Figure 1. Developmental defects and microphthalmia in *Gsk3*-deficient retina with aberrant nuclear translocation of β-catenin, a key effector of the Wnt canonical pathway. (A) Immunohistochemistry (IHC) of E12.5 retina from *Gsk3α$^{f/f}$β$^{f/f}$* mice expressing or not α-Cre using a pan-GSK3 antibody (green) shows efficient deletion at the periphery where the Cre expression has been previously reported (delimited by dashed-line) Scale bar: 100 μm. (B) Hematoxylin and eosin (H&E) staining on methacrylate sections at E12.5, E14.5 and P2 reveals large retinal morphogenesis defects in *Gsk3α$^{f/f}$β$^{f/f}$;α-Cre* with blood invasion into the eyeball (showed by white arrow). L, Lens; NR, neural retina. Scale bar: 100 μm at E12.5 and E14.5. 500 μm at P2. For the magnification of P14.5, scale bar: 50 μm.

Figure 2. One allele of either *Gsk3α* or *Gsk3β* is sufficient for the development of a functional retina. (A) Immunoblot analysis of protein extracts from 2-month-old animals with different combination of *Gsk3α* and *Gsk3β* floxed alleles (*Gsk3α$^{f/f}$β$^{+/+}$*, *Gsk3α$^{+/+}$β$^{f/f}$*, *Gsk3α$^{f/+}$β$^{f/f}$* or *Gsk3α$^{f/f}$β$^{f/+}$*) with or without *Cre* recombinase using anti-panGSK3 antibody (recognizing both isoforms) reveals decreased expression of GSK3α or GSK3*β* (arrowheads). *α*-Tubulin is used as loading control. (B) IHC on 2-month-old retinal sections from control and *Gsk3α$^{f/+}$β$^{f/f}$*; *α-Cre* retinas with or without Cre recombinase using anti-GSK3β antibody (red) showing ubiquitous *Gsk3β* expression in all retinal layers, whereas its expression is lost in the Cre-expressing retina. (C) Expression of only one *Gsk3* allele (*Gsk3α*) is sufficient for proper photoreceptor development. IHC using anti-Rhodopsin (Rho, red) and anti-Cone arrestin (Arr3, red) antibodies to label rod and cone photoreceptors, respectively. (D) Expression of only one *Gsk3* allele (*Gsk3α*) is sufficient for proper interneuron

development. IHC using anti-Calretinin (Calr, green) and anti-Calbindin (Calb, red) antibodies to label horizontal and amacrine cells, respectively. (B-D) onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 20μm. (E, F) Electroretinogram (ERG) recording in 2-month-old *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* animals and littermate controls. Photopic (cones) (E) and scotopic (rods) (F) response in *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* animals are similar to controls. Mean ± SEM intensity response curves of a- and b-wave responses averaged from 8 biological replicates of each genotype.

Figure 3. Gradual loss of *Gsk3α* and/or *Gsk3β* 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 *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* mouse retina reveals the presence of supernumerary displaced retinal ganglion cells (dRGCs, arrows) in the INL of *Gsk3α$^{f/+}$β$^{f/f}$; α-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) dRGCs send their axons into the optic nerve. Visualization of dRGCs after 3D reconstruction of 2-month-old flat mounted retina of control and *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* animals following retrograde labeling with Rhodamin-Dextran applied onto the optic nerve. inl: inner nuclear layer, gcl: ganglion cell layer. (C) Gradual loss of *Gsk3α* and *Gsk3β* alleles (*Gsk3α$^{f/f}$β$^{+/+}$, Gsk3α$^{+/+}$β$^{f/f}$, Gsk3α$^{f/+}$β$^{f/f}$* or *Gsk3α$^{f/f}$β$^{f/+}$*) leads to a gradual increase of Brn3a-positive RGCs located to the INL, with the highest number observed in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* animals. ~~Left~~ Histograms represent counting of the total number of Brn3a-positive cells per section located in the GCL (*left panel*) or in the INL (*middle panel*). Right histogram represents the percentage of the dRGCs among the total number of Brn3a-positive cells per section for each combination. Mean ± 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 *Gsk3α*$^{f/+}$*β*$^{f/f}$*; α-Cre* dRGCs and in littermate
821 controls. Scale bar: 20 μm. (E) Flat mounted retina from *Gsk3α*$^{f/+}$*β*$^{f/f}$*; α-Cre* and
822 littermate controls labelled with anti-Rbpms antibody demonstrated the large number
823 of Rbpms-positive dRGCs in the INL of *Gsk3α*$^{f/+}$*β*$^{f/f}$*; α-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 *Gsk3α*$^{f/+}$*β*$^{f/f}$; *α-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 ±
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 *Gsk3α*$^{f/+}$*β*$^{f/f}$*; α-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 *Gsk3α*$^{f/+}$*β*$^{f/f}$*; α-Cre* retina. Right histogram represents the percentage of the dRGCs
841 among the total number of Brn3a-positive cells per section. Mean ± SEM values are
842 presented from 6 biological replicates. A nonparametric Mann-Whitney U test was

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

Figure 5. Lack of Gsk3β results in RGC projections into the ipsilateral Medial Terminal Nucleus. (A) All panels are light sheet fluorescence microscopy of solvent-cleared adult brain from control, *Gsk3α$^{f/f}$β$^{+/+}$; α-Cre*, *Gsk3α$^{+/+}$β$^{f/f}$; α-Cre* and *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* animals after intravitreal injection of CTB coupled to either an Alexa-555 or -647. Ipsilateral projections of RGCs into the MTN was observed in the absence of *Gsk3β* expression. SC, superior colliculus; NOT, nucleus of optic tract; dLGN, dorsal lateral geniculate nucleus; vLGN, ventral lateral geniculate nucleus; IGL, intergeniculate leaflet; OPT, Olivary Pretectal Nucleus; dMTN, dorsal medial terminal nucleus; MTN, medial terminal nucleus; vMTN, ventral medial terminal nucleus; OT, Optic tract; SCN, suprachiasmatic nucleus; ON, optic nerve. Scale bar: 1mm; * indicates the ipsilateral MTN. (B) Quantification of the signal intensity ratio between ipsilateral and contralateral MTN in controls and *Gsk3* mutants (including *Gsk3α$^{f/f}$β$^{+/+}$; α-Cre*, *Gsk3α$^{+/+}$β$^{f/f}$; α-Cre*, and *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre*). A nonparametric Mann-Whitney U test was applied, ns: non-significant, ** $P \leq 0.01$. See Extended Data Figure 5-1 for co-staining of the CTB-positive cells with Brn3a and NF68.

Figure 6. Whole transcriptome meta-analysis suggests that dRGC in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina are direction-selective ganglion cells. (A) Volcano plot representation of differentially expressed genes between *Gsk3αf/+βf/f; α-Cre* and control retina plotted on the x-axis (log2 scale). FDR adjusted significance is plotted on the y-axis. Orange and blue dots: significantly up-regulated and down-regulated genes in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina, respectively. Vertical dashed lines represent FC=1.5. Horizontal dashed line represents FDR=0.05. (B) Chord plot representation

of DEGs related to GO annotations belonging to either molecular functions (MF) or biological process (BP). Overlaps in GO annotation amongst genes within each category are visualized. * correspond to genes expressed in previously published purified RGCs (blue, slightly expressed genes in RGCs between 1 and 5 FPKM; red, 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-qPCR of *Cartpt, Th, Epha2, Cplx1, Chrna5, Chrna2, Chrna7, Chrnb4* in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina at 2-months of age, relative to littermate control retina levels. All values are expressed as the Mean ± SEM from three biological replicates. A nonparametric Mann-Whitney U test was applied, * indicates $P \leq 0.05$. (D) IHC on 2-month-old mouse retina reveals the presence of a subset of dRGCs (Rbpms-positive dRGCs, red) in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* expressing either the transcription factor Tbr2 (grey) or Foxp2 (green). Arrows indicate Tbr2 and Rbpms-positive dRGCs; arrowheads represent Foxp2 and Rbpms-positive dRGCs. onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 50 µm. (E) The mean OMR indices (±SEM) are plotted as a function of spatial frequency for each genotype (n=13 for *Gsk3α$^{f/+}$β$^{f/f}$* and 18 for *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* genotype). The baseline (1; dashed line) represents unspecific head movements and no response to the stimulus. OMR at 100% and 50% contrast in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* mice (dashed line) and controls (black line). A Grubbs test was performed at 5% to remove outliers followed by 2-way ANOVA, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$. See Extended Data Figure 6-1 for the hierarchical clustering of the DEGs. See Extended Data Figure 6-2 for pathway analysis results.

EXTENDED DATA

Figure 3-1. dRGCs express the nuclear factor Islet-1. (A) IHC on 2-month-old mouse retina reveals that most dRGCs (Rbpms-positive dRGCs, white arrows, red) in the INL of *Gsk3α$^{f/+}$β$^{f/f}$; α-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. onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar: 50 µm. (B) Counting on flat mount of Rbpms- or Brn3a- positive cells located in the INL at the dorsal, ventral, nasal and temporal part of control and *Gsk3α$^{f/+}$β$^{f/f}$;α-Cre* retina. Histogram represents the number of Brn3a- or Rbpms-positive cells per field. Mean ± SEM values are presented from 4 biological replicates.

Figure 3-2. Brn3a-positive cells located in the INL of *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina are dRGCs. Brn3a-positive RGCs located in the INL of *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina do not express markers of other INL neurons such as Choline-Acetyltransferase (Chat) or Calbindin (Calb). onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Arrowheads indicates Brn3a-positive dRGCs. Scale bar: 20µm.

Figure 5-1. Intravitreal injection of CTB labels dRGCs. After intravitreal injection of CTB coupled to an Alexa-555 (*red*) in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* eye led to the labelling of Brn3a- (green) and NF68-positive (*grey*) cells located in the INL. Scale bars: 20µm

Figure 6-1. Hierarchical clustering of the identified differentially expressed genes. Hierarchical clustering representing the 111 DEGs (abs(FC)≥1.5; FDR≤0.05; FPKM>1) between 2-month-old *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina and littermate control were clustered by their Z-score. Each column for each genotype corresponds to one

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

Figure 6-2. Identification of enriched pathways from DEGs identified in 2-month-old *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina. (A) Gene ontology (GO) annotations of DEGs in *Gsk3α$^{f/+}$β$^{f/f}$; α-Cre* retina compared to littermate controls. Top over-represented pathways for Biological process (BP), Molecular Function (MF), KEGG (Kyoto Encyclopedia of Genes and Genomes) and TRRUST (Transcriptional Regulatory Relationships Unrevealed by Sentence-based Text mining) were identified by enrichment analysis using Metascape. (B) Circular visualization for BP and MF of GO enrichment analysis. Down-regulated genes (blue dots) and up-regulated genes (red dots) within each GO pathway are plotted based on logFC. Z-score bars indicate if an entire GO category is more likely to be increased or decreased based on the genes within it.

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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)
α-tubulin	mouse	SIGMA	T5168		1:200.000
GSK3α/β	mouse	Thermo Fisher Scientific	44-610	1:250	1:1000
GSK3β	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	

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954 **Secondary antibodies**

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

anti-mouse IgG2B		Scientific		
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

Gene name	Primer F	Primer R
Cartpt	5'-TAAAGTTTGCGTTCCCCTCAG-3'	5'-CAACACCATTCGAGGCATTCT-3'
Th	5'-ACTATGCCTCTCGTATCCAGC-3'	5'-CGGATGGTGTGAGGACTGTC-3'
Epha2	5'-GACCTCCCCATCTTCATTTGG-3'	5'-GCGTACAGTGCCCTAGTCATA-3'
Cplx1	5'-GGTGATGAGGAAAAGGACCCC-3'	5'-TCTTGGCGTACTTTGCTTTGC-3'
Chrna5	5'-CTTGAGTACCAACACTGTCCG-3'	5'-CCAGTACTCCAAAGATGCCCT-3'
Chrna2	5'-CATTATCGTCTGCTTCCTGGG-3'	5'-CTTGGAGCCAACATGAGGGA-3'
Chrna7	5'-CTGTAGCTGTCGGTCTTGAGA-3'	5'-CAATGATATGCCGGTGATGGG-3'
Chrnb4	5'-AAACTGATCTGGCTACCTCCC-3'	5'-GTAGAGAGTCCAGGAGATGCC-3'

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

Table 2. List of primers used for RT-qPCR