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RESEARCH REPORT

The LGN protein promotes planar proliferative divisions in the neocortex but apicobasal asymmetric terminal divisions in the retina

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ABSTRACT

Cell division orientation is crucial to control segregation of polarized fate determinants in the daughter cells to produce symmetric or asymmetric fate outcomes. Most studies in vertebrates have focused on the role of mitotic spindle orientation in proliferative asymmetric divisions and it remains unclear whether altering spindle orientation is required for the production of asymmetric fates in differentiative terminal divisions. Here, we show that the GoLoco motif protein LGN, which interacts with Gαi to control apicobasal division orientation in *Drosophila* neuroblasts, is excluded from the apical domain of retinal progenitors undergoing planar divisions, but not in those undergoing apicobasal divisions. Inactivation of LGN reduces the number of apicobasal divisions in mouse retinal progenitors, whereas it conversely increases these divisions in cortical progenitors. Although LGN inactivation increases the number of progenitors outside the ventricular zone in the developing neocortex, it has no effect on the position or number of progenitors in the retina. Retinal progenitor cell lineage analysis in LGN mutant mice, however, shows an increase in symmetric terminal divisions producing two photoreceptors, at the expense of asymmetric terminal divisions producing a photoreceptor and a bipolar or amacrine cell. Similarly, inactivating Gαi decreases asymmetric terminal divisions, suggesting that LGN function with Gαi to control division orientation in retinal progenitors. Together, these results show a context-dependent function for LGN and indicate that apicobasal divisions are not involved in proliferative asymmetric divisions in the mouse retina, but are instead essential to generate binary fates at terminal divisions.

KEY WORDS: Asymmetric cell division, Retina, Cell lineage, Oriented divisions, Cell fate decision, Stem cell, Self-renewal, Differentiation

INTRODUCTION

Whereas differential exposure to environmental cues can instruct asymmetric fates, varying mitotic spindle orientation can produce intrinsically asymmetric cell divisions by segregating fate determinants unequally in the daughter cells (Siller and Doe, 2009). Extensive work over the past several years has provided detailed insights on the mechanisms regulating spindle orientation in *Drosophila* neuroblasts (Gönczy, 2008; Knoblich, 2008; Yu et al., 2006). The polarity protein Par-3 interacts with the adaptor protein Inscuteable (Insc), which, in turn, recruits Partner of Inscuteable (Pins) to the apical cortex. Pins then interacts with cortical Gαi-GDP and with the microtubule binding protein Mud. Together, this complex recruits dynein on astral microtubule, providing the necessary force to position the mitotic spindle along the apicobasal axis (Bowman et al., 2006; Du and Macara, 2004; Du et al., 2001; Izumi et al., 2006; Schaefer et al., 2000; Siller et al., 2006). The homologs of Pins (AGS3 and LGN, also known as GPSM1 and GPSM2, respectively), Gαi (GNAI1-GNAI3) and Mud (NUMA1), have also been involved in the control of mitotic spindle orientation in various tissues in vertebrates (Lu and Johnston, 2013; Morin and Bellaïche, 2011).

In the developing central nervous system, the importance of division orientation in the production of asymmetric cell divisions remains a matter of debate (Lancaster and Knoblich, 2012). Although some studies in the developing neocortex and neural tube provided evidence that apicobasal divisions trigger asymmetric proliferative divisions producing a progenitor cell and a neuron (Alexandre et al., 2010; Das and Storey, 2012; Xie et al., 2013), others have suggested that apicobasal divisions do not play a part in cell fate decisions, but instead control the position of progenitors in the neuroepithelium (Konno et al., 2008; Morin et al., 2007), which was later proposed to control the balance between direct and indirect neurogenesis in the developing neocortex (Postiglione et al., 2011). In these studies, divisions generating two neurons ('terminal' divisions) were generally considered symmetric, even though they are *de facto* asymmetric when the neuronal cells produced are of different types.

In the developing rat retina, retinal progenitor cells (RPCs) divide almost exclusively with their mitotic spindle parallel to the plane of the neuroepithelium (horizontal) at early stages when divisions are mostly proliferative (Cayouette et al., 2001), arguing against a role for division orientation in asymmetric proliferative divisions. At late stages of retinogenesis, however, when many divisions are terminal, some RPCs reorient their spindle to divide at perpendicular angles (vertical) relative to the neuroepithelium (Cayouette et al., 2001). Live imaging experiments have shown that such vertical divisions are terminal and largely correlate with the production of two differentiating daughter cells that acquire different fates, whereas terminal divisions with a horizontal spindle correlate with the production of two differentiating daughter cells that acquire the same fate (Cayouette and Raff, 2003). It remains unclear, however, whether division orientation simply correlates with, or actually determines asymmetric outcome in RPC terminal divisions. Here,

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Development (2016) 143, 575-581 doi:10.1242/dev.129783

we address this question by altering spindle orientation using genetic inactivation of LGN in RPCs.

RESULTS AND DISCUSSION

Localization of LGN varies depending on division orientation in RPCs

Because LGN is a key member of the spindle orientation complex, we hypothesized that it might be involved in regulating the orientation of RPC division. Consistently, we found abundant expression of *Lgn* and *Ags3* in the progenitor layer (Fig. 1A-F). We next studied LGN protein localization in RPCs at P0. In interphase RPCs, LGN was detected around the plasma membrane, but was enriched at the apical domain (Fig. 1G). In mitosis, LGN labeling was mostly concentrated at the lateral poles and excluded from the apical domain in horizontal divisions (Fig. 1I,I′,K), whereas it invaded the apical domain in oblique and vertical divisions (Fig. 1J-K). Because LGN antibodies also recognize the LGN homolog AGS3 (Konno et al., 2008 and Fig. S1), we wanted to provide additional evidence for the localization of LGN itself in RPCs. To do this, we electroporated a construct coding for an EGFP::LGN fusion protein in P0 retinas *in vivo* and studied its localization 24 h later. Consistent with immunostaining results, EGFP::LGN was enriched at the apical domain in interphase RPCs (Fig. 1L,M), and localized to the lateral poles but not the apical domain in horizontal mitotic RPCs, whereas it was found apically in vertical mitotic RPCs (Fig. 1N-P). These results suggest that LGN might be required at the apical domain to instruct vertical divisions.

LGN is required for vertical spindle orientation in mouse RPCs

To study LGN function in spindle orientation, we analyzed P0 retinas of a LGN knockout mouse ($Lgn^{-/-}$) that we previously generated (Tarchini et al., 2013) and used heterozygote littermates

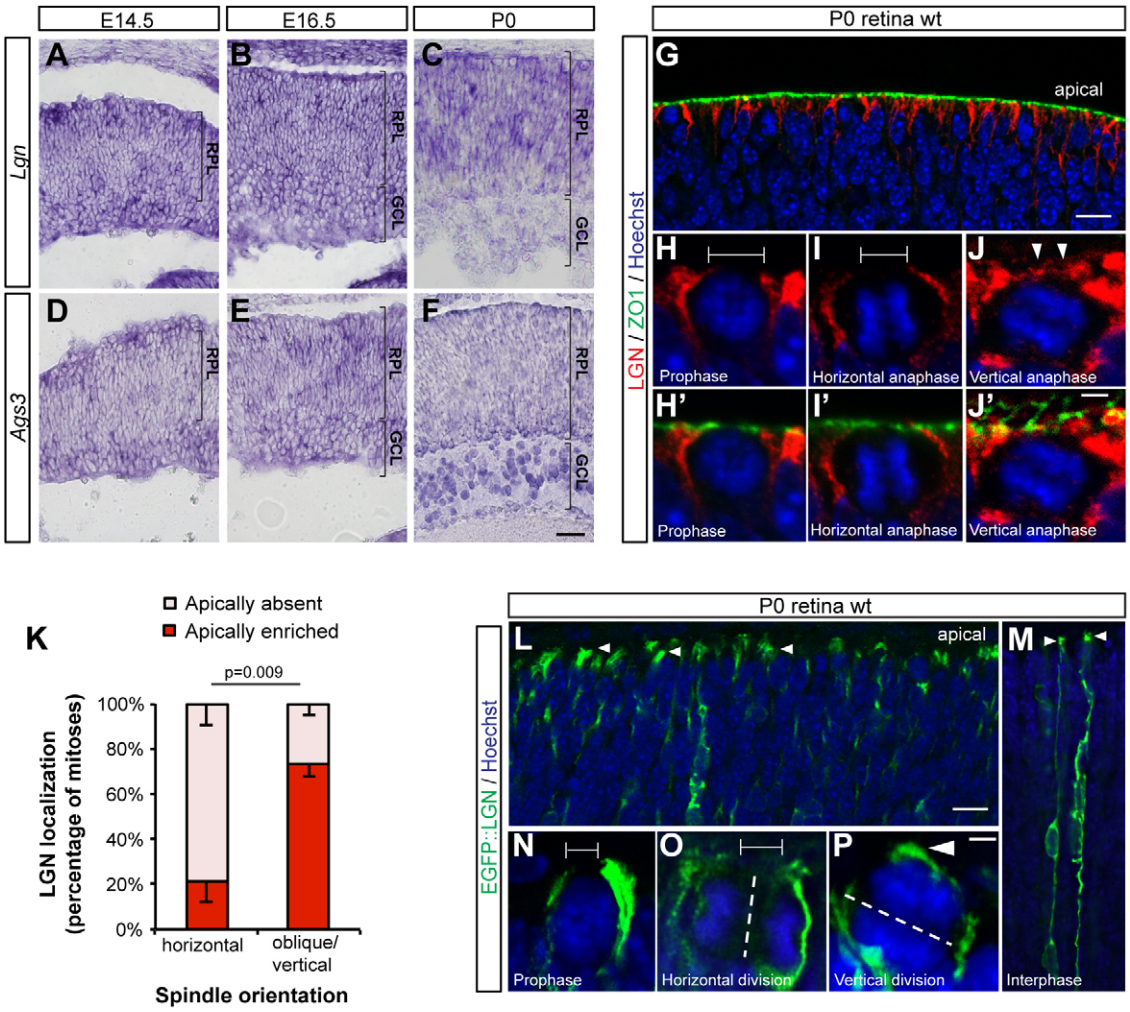

Fig. 1. LGN is expressed in the retina. (A-F) *In situ* hybridization on transverse retinal sections with *Lgn* (A-C) and *Ags3* (D-F) antisense probes. E, embryonic day; P, postnatal day; RPL, retinal progenitor cell layer; GCL, ganglion cell layer. (G-J) Immunofluorescence staining for LGN (red) and the apical junction marker ZO-1 (green) in wild-type mouse retina at P0. In prophase (H,H′) and horizontal anaphase/telophase cells (I,I′), LGN is localized to the basolateral membrane and absent from the apical membrane (brackets), as defined by the space between the ZO-1-labeled junctions. In vertical and oblique anaphase RPCs (J,J′), LGN is detected at the apical membrane (arrowheads). In J′, the ZO-1 staining observed at the top of the panel arises from the apical membrane of the retinal pigment epithelium, which remains attached to the retina in this example. (K) LGN localization in anaphase/telophase RPCs as a function of spindle orientation. Data presented as means±s.e.m. (n=47 horizontal divisions and n=35 oblique/vertical divisions from 3 mice). (L-P) EGFP::LGN localization 24 h after electroporation in P0 retinas *in vivo*. In interphase RPCs (L,M), EGFP::LGN is enriched at the apical domain (arrowheads). In mitotic RPCs (N-P), EGFP::LGN localizes to the basolateral membrane in prophase and horizontal anaphase (brackets), but invades the apical domain in vertical anaphase cells (arrowhead). Dashed lines in O and P indicate cleavage planes. Scale bars: 50 µm in A-F, 10 µm in G,L and 2 µm in H-J and N-P. In all images, apical side faces up.

Development (2016) 143, 575-581 doi:10.1242/dev.129783

($Lgn^{+/-}$) as controls. We measured the angle of the mitotic spindle by tracing a line between the two centrosomes in 3D, relative to the plane of the apical surface (see Materials and Methods and Fig. 2A,B).

In controls, we found that most divisions were horizontal (≤30°), whereas 33% were either oblique (30-60°) or vertical (>60°) (Fig. 2C). These numbers are similar to those obtained in wild-type retinas (Cayouette and Raff, 2003; Cayouette et al., 2001), suggesting that the loss of one copy of LGN in heterozygotes does not significantly alter spindle orientation. In $Lgn^{-/-}$ mice, however, the proportions of oblique and vertical divisions were reduced by more than half (Fig. 2C). Thus, much like in *Drosophila* neuroblasts and mouse skin epithelial cells (Schaefer et al., 2000; Williams et al., 2014; Yu et al., 2000), LGN is required to promote vertical spindle orientation in mouse RPCs. These results also indicate that LGN is not absolutely required for horizontal divisions, suggesting that the absence of LGN at the apical membrane in horizontal divisions is functionally more important than its presence at the lateral poles in RPCs. Because AGS3 is expressed in RPCs, and was shown to control spindle orientation in cortical progenitors (Sanada and Tsai, 2005), it is possible that AGS3 compensates for LGN inactivation. It will be interesting in the future to generate double-knockout animals to investigate this possibility. Additionally, identifying the mechanisms operating to exclude LGN from the apical domain will be important to understand how horizontal divisions are generated in RPCs.

Inactivation of LGN decreases apical progenitors in the developing neocortex but not the retina

Our results point to a role for LGN in promoting vertical divisions in the retina, which is in sharp contrast to previously published data in the mouse neocortex and chick neural tube, where LGN is required to maintain horizontal divisions (Konno et al., 2008; Morin et al., 2007). We therefore wondered whether this discrepancy could be due to tissue-specific functions of LGN or could be explained by differences in the mouse mutants used (Konno et al., 2008; Tarchini et al., 2013). As previously reported (Konno et al., 2008), we found that spindle orientation of cortical progenitors was randomized in our $Lgn^{-/-}$ mouse model at embryonic day (E)14.5 (Fig. 2D). Thus, in the same mouse model, LGN inactivation decreases or increases vertical divisions in retinal and cortical progenitors, respectively, suggesting tissue-specific functions.

We next wondered whether the alterations in spindle orientation observed in the developing neocortex and retina could affect the number and distribution of proliferating cells. As previously reported (Konno et al., 2008), we observed an important increase in BrdU-positive and pH3-positive cells outside the ventricular zone of $Lgn^{-/-}$ cortices at the expense of apical progenitors (Fig. 3A-F), confirming a role for LGN in maintaining planar divisions and self-renewal of cortical progenitors. When we analyzed proliferation in the mouse retina, however, we did not observe significant changes in the number or location of BrdU-positive and pH3-positive cells in $Lgn^{-/-}$ compared with controls at both E14.5 and P0 (Fig. 3G-M and Fig. S2). Thus, inactivation of LGN does not affect proliferative divisions in the mouse retina, suggesting that they are regulated independently of division orientation.

LGN is required for terminal asymmetric cell divisions in the mouse retina

Because altering division orientation did not affect proliferation in the retina, we hypothesized that it could be involved in the production of symmetric or asymmetric terminal divisions. To directly address this question, we used a GFP retroviral vector to clonally mark RPC lineages in retinal explants prepared from $Lgn^{+/-}$ or $Lgn^{-/-}$ mice at postnatal day (P)0. Fourteen days later, when all the cells had differentiated, we studied the cellular composition of the resulting clones using morphology, layer position, and cell type specific marker expression (Fig. 4A). When we analyzed the overall size distribution of the clones produced in $Lgn^{-/-}$ retinas compared with their control littermates, we found no difference (Fig. 4B), further supporting our conclusion that changing spindle orientation does not affect proliferation or cell death in the retina.

To more directly study the outcome of terminal divisions, we focused our analysis on two-cell clones. Since naturally occurring cell

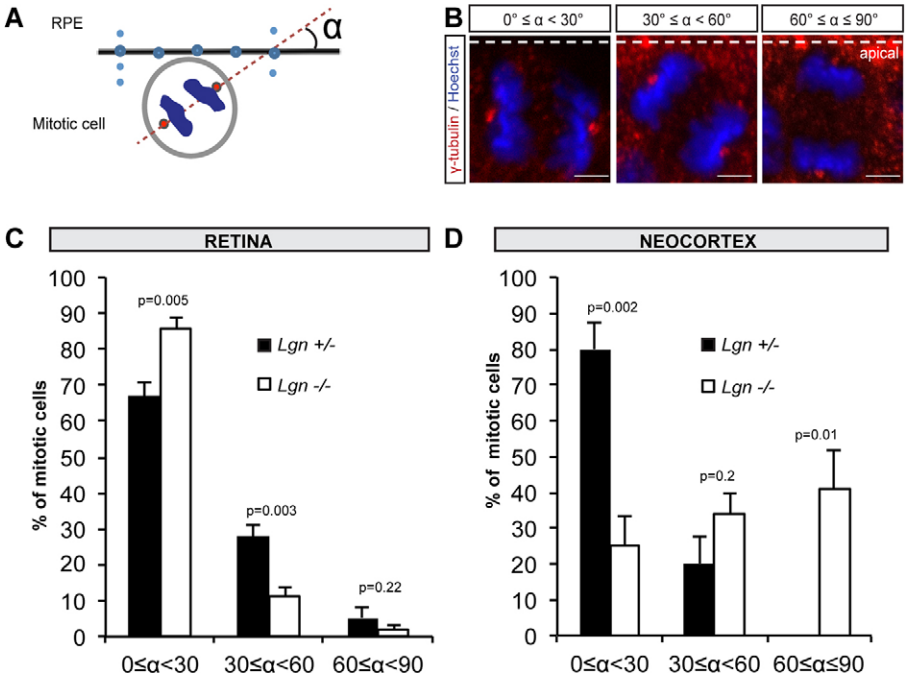

Fig. 2. LGN inactivation decreases vertical divisions in the retina but randomizes division orientation in the neocortex. (A) Cartoon illustrating the method used to calculate mitotic spindle orientations. Centrosomes are shown in red and DNA in blue. The plane of the apical surface is obtained by defining several coordinates (blue dots) in 3D along the interface between the retinal pigment epithelium (RPE) and the neural retina. The angle (α) of the mitotic spindle is then calculated using standard trigonometry. (B) Examples of horizontal (0-30°), oblique (30-60°) and vertical divisions (60-90°). Sections were stained for γ-tubulin (red) and Hoechst (blue). Scale bars: 1 µm. (C) Quantification of mitotic spindle orientation in P0 RPCs in 4 $Lgn^{+/-}$ (n=256 cells analyzed) and 6 $Lgn^{-/-}$ (n=358 cells analyzed). (D) Quantification of mitotic spindle orientation in E14.5 forebrain progenitors from 3 $Lgn^{+/-}$ mice (n=69 cells analyzed) and 4 $Lgn^{-/-}$ mice (n=102 cells analyzed). In C and D, data are presented as means ±s.e.m., two-tailed t-test.

Development (2016) 143, 575-581 doi:10.1242/dev.129783

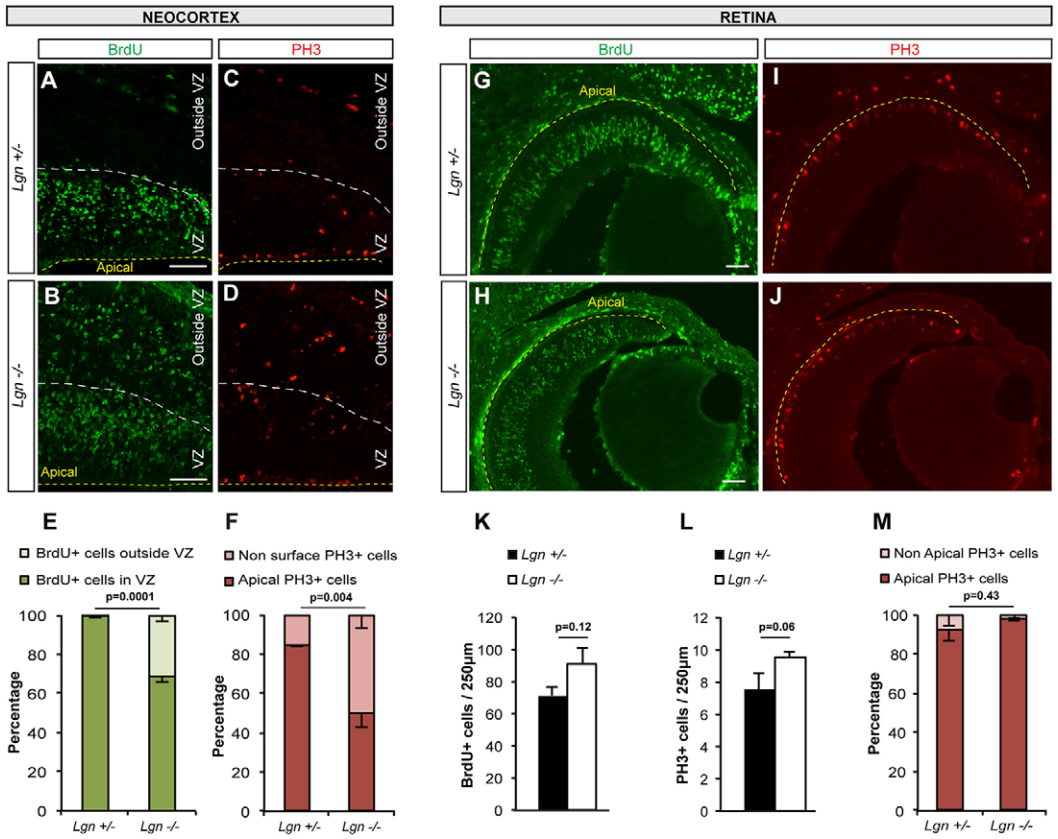

Fig. 3. LGN inactivation increases non-surface progenitors in the developing neocortex but not the retina. (A-D) Immunostaining for BrdU (A,B) and pH3 (C,D) in frontal sections of E14.5 forebrain from $Lgn^{+/-}$ (A,C) and $Lgn^{-/-}$ (B,D) mice. VZ, ventricular zone. The yellow dashed line indicates the apical surface and the white dashed line marks the basal side of the VZ. Scale bar: 50 µm. (E,F) Quantification of $BrdU^{+}$ and $pH3^{+}$ cells in control (n=4) and $Lgn^{-/-}$ forebrain (n=4) at E14.5 (data obtained from 3 independent experiments, presented as means±s.e.m., two-tailed t-test). (G-J) Immunostaining for BrdU and pH3 on E14.5 retinal sections from $Lgn^{+/-}$ and $Lgn^{-/-}$ mice, as indicated. The yellow dashed line indicates the apical surface. Scale bar: 50 µm. (K-M) Quantification of $BrdU^{+}$ and $pH3^{+}$ cells in $Lgn^{+/-}$ (n=4) and $Lgn^{-/-}$ (n=4) mice at E14.5 (data obtained from 4 independent experiments, presented as means±s.e.m., two-tailed t-test).

death is limited in the developing retina (Young, 1984), we assume that these divisions are generated by RPCs undergoing a terminal division producing two differentiating daughter cells. Remarkably, we found that terminal asymmetric divisions were reduced by more than half in absence of LGN ($Lgn^{+/-}$, 23.5%; $Lgn^{-/-}$, 10%), whereas terminal symmetric divisions were increased accordingly ($Lgn^{+/-}$, 76.5%; $Lgn^{-/-}$, 90%). More specifically, terminal asymmetric divisions giving rise to a photoreceptor and a bipolar or an amacrine cell were reduced in $Lgn^{-/-}$, whereas terminal symmetric divisions giving rise to two photoreceptors were increased (Fig. 4C). Interestingly, the proportions of symmetric and asymmetric terminal divisions observed in $Lgn^{-/-}$ retinas and controls are consistent with the respective proportions of horizontal and vertical divisions observed in each genotype (compare Fig. 2C and Fig. 4C). We therefore conclude that, in the mouse retina, vertical spindle orientation is required for the production of terminal asymmetric divisions. In a previous study, we showed that the endocytic adaptor protein NUMB localizes apically in RPCs and is essential for the production of terminal asymmetric cell divisions (Kechad et al., 2012). Because we did not detect any change in NUMB localization in the $Lgn^{-/-}$ retina (Fig. S3), we propose that the increased number of horizontal divisions observed in $Lgn^{-/-}$ retinas promotes symmetric inheritance of NUMB, which increases symmetric terminal divisions at the expense of asymmetric terminal divisions.

As we find that reorientation of the mitotic spindle is only involved in the production of terminal asymmetric divisions, we predicted that this would not have much effect on the overall proportions of each cell type in the mature retina. Indeed, when we used the data from retinal lineages that we reconstructed in a previous study (Gomes et al., 2011) and changed the composition of asymmetric terminal divisions to a symmetric pair of photoreceptors in 13% of the cases, which represent the changes observed in $Lgn^{-/-}$ retinas (see Fig. 4C), we found that this would result in only a small percentage difference in overall cell type composition (Fig. S4A). Consistent with this modelization, we did not detect significant changes in overall cell type proportions in the $Lgn^{-/-}$ retinas compared with controls (Fig. S4B-K). Although re-orientation of the mitotic spindle in terminal divisions might not drastically affect the overall distribution of cell types, it might allow the formation of functional circuits between sister cells, which were shown to preferentially make synaptic contact with each other in the neocortex (Yu et al., 2009).

We next wondered whether Gαi, a well-known partner of LGN, was involved in controlling spindle orientation in RPCs. We found that at least two of the three Gαi mouse genes (*Gnai2* and *Gnai3*), as well as Gαo (*Gnao1*), are expressed in the retina at P0 (Fig. 4D,E). To inactivate G protein signaling, we used a retroviral construct encoding the catalytic subunit of pertussis toxin (PTXa) and GFP from an internal ribosome entry site (Peyre et al., 2011). In a previous study, we established that PTXa impairs the co-immunoprecipitation of LGN with Gαi and delocalizes LGN from the cell cortex (Tarchini et al., 2013). Similarly, we found that PTXa delocalized LGN from RPC membranes (Fig. S4L). If LGN requires interaction with Gαi to

Development (2016) 143, 575-581 doi:10.1242/dev.129783

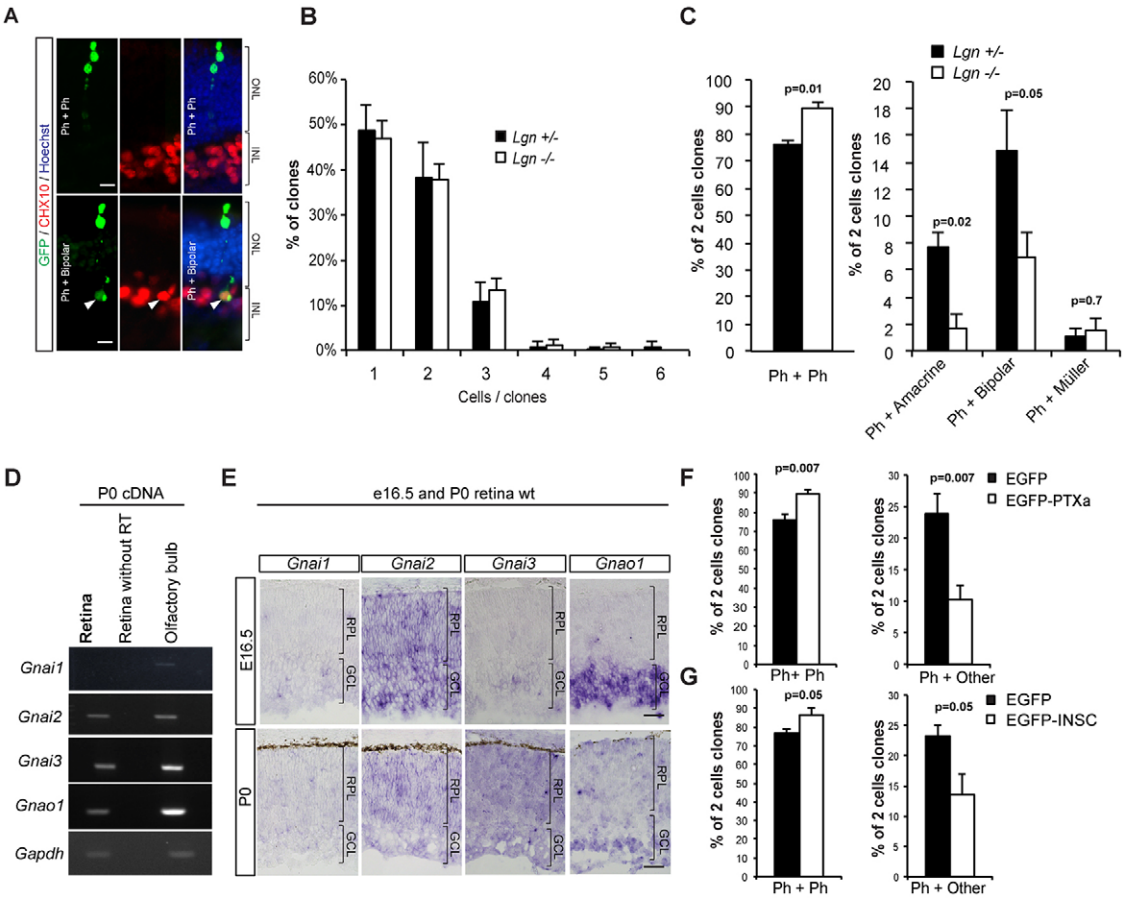

Fig. 4. LGN and Gαi activity are required for terminal asymmetric cell divisions in the retina. (A) Examples of two-cell clones obtained 14 days after infection of $Lgn^{+/-}$ or $Lgn^{-/-}$ retinal explants at P0 with a GFP retroviral vector. Retinal sections are stained with CHX10 to identify bipolar cells (arrowhead). Ph, photoreceptor; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 5 µm. (B) Average number of cells per clones in 4 $Lgn^{+/-}$ (*n*=450 clones) and 5 $Lgn^{-/-}$ (*n*=599 clones) mice from 4 independent experiments (means±s.e.m.). The clone size distribution is not significantly different between conditions. (C) Composition of two-cell clones in 4 $Lgn^{+/-}$ (*n*=172 two-cell clones) and 5 $Lgn^{-/-}$ (*n*=219 two-cell clones) mice from 4 independent experiments (means±s.e.m., two-tailed *t*-test). (D) RT-PCR at P0 for *Gnai1-Gnai3* and *Gnao*. RT, reverse transcription. (E) *In situ* hybridization for *Gnai1-Gnai3* and *Gnao* on retinal sections. Scale bars: 50 µm for P0 and 150 µm for E16.5. RPL, retinal progenitor cell layer; GCL, ganglion cell layer. (F,G) Composition of two-cell clones 14 days after infection with retroviral vectors expressing a control EGFP compared with EGFP-PTXa (F) or EGFP-INSC (G). Data presented as means±s.e.m.; two-tailed *t*-test. In F, *n*=341 two-cell clones for EGFP and *n*=248 two-cell clones for EGFP-PTXa, from 5 independent experiments. In G, *n*=274 two-cell clones for EGFP and *n*=259 two-cell clones for EGFP-INSC, from 4 independent experiments.

reorient the mitotic spindle, we predicted that PTXa would phenocopy LGN inactivation. Consistently, we found that terminal symmetric divisions were increased, whereas terminal asymmetric divisions were reduced upon inactivation of Gαi (Fig. 4F), suggesting that LGN functions together with Gαi to promote vertical divisions in RPCs. Similarly, overexpression of mouse inscuteable (INSC), another key regulator of division orientation, phenocopied Gαi and LGN inactivation (Fig. 4G). Thus, much like in the neocortex, INSC overexpression produces a similar phenotype to LGN inactivation (Konno et al., 2008; Postiglione et al., 2011), although in the retina their effect on division orientation is apparently opposite, suggesting that different mechanisms are at play.

In conclusion, this study demonstrates a key role for mitotic spindle orientation in the production of symmetric and asymmetric terminal divisions in the mouse retina, and establishes LGN and Gαi as key regulators in this context. Importantly, we report that inactivation of LGN disrupts spindle orientation in both the developing neocortex and retina, but this leads to very different outcomes. What exactly triggers different fate decisions after reorientation of the mitotic spindle in the neocortex and retina remains unclear, but it is likely to involve segregation of different fate determinants and a tight control over the timing of spindle reorientation. It will be interesting in the future to determine why the spindle appears to reorient only at terminal divisions in the retina, whereas it reorients in proliferative divisions in the developing neocortex. This is likely to involve novel regulators of LGN activity or localization that are tissue dependent and temporally restricted.

MATERIALS AND METHODS

Animals

All animal work was performed in accordance with the Canadian Council on Animal Care guidelines. CD1 and LGN mutants of either sex were used in this study.

Cell lines

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin-streptomycin (Invitrogen) in a 37°C humidified incubator with 5% carbon dioxide.

Mitotic spindle orientation

Mitotic spindle orientation was measured as previously described (Cayouette et al., 2001), with the exception that a 3D plane, instead of a

Development (2016) 143, 575-581 doi:10.1242/dev.129783

line, was used as the reference, as reported (Juschke et al., 2014). Further details can be found in the supplementary Materials and Methods.

Retinal explant culture

P0 retinal explants were prepared and infected with retroviruses as previously described (Cayouette et al., 2001). The explants were infected with a retroviral vector encoding green fluorescent protein (LZRS, pBird, pBird-PTXa or pBird-INSC). Retroviral vectors were prepared in Phoenix packaging cell line and used to infect explants as described previously (Cayouette and Raff, 2003).

Clonal analysis

After 14 days in culture, the explants were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature and processed for immunostaining. Clones derived by each infected retinal progenitor cells were analyzed by counting the number of each cell type present in radial clusters, using morphology, position in the cell layers and expression of CHX10 or PAX6 as a marker of bipolar cells and amacrine cells, according to standard procedures as previously described (Elliott et al., 2008; Kechad et al., 2012).

In situ hybridization

Digoxigenin-labeled RNA probes were synthesized from pSport6-LGN, pGEMTeasy-AGS3, pGemteasy-Gαo, pGemteasy-Gαi1, pGemteasy-Gαi2 and pGemteasy-Gαi3. Eyes and head were fixed in 4% PFA overnight, frozen in OCT and sectioned at 12 µm. Sections were incubated with hybridization buffer at 65°C and with 100 ng/ml RNA probe in hybridization buffer (50% formamide, 5× saline-sodium citrate, 5× Denhardt's, 5 mg/ml Torula RNA). The probes were detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2500, Roche). The AP activity was revealed using 4-nitro blue tetrazolium chloride (NBT/BCIP; Roche).

Histology and immunofluorescence

Eyes were enucleated in PBS and fixed by immersion in freshly prepared 4% PFA in PBS, or in 10% trichloroacetic acid (TCA) diluted in water for LGN staining. Eyes were cryoprotected overnight at 4°C in sucrose 20%, embedded in 20% sucrose:OCT (1:1), and stored at −80°C until sectioning. Mouse brains were fixed in 4% PFA in phosphate buffer at 4°C following by cryoprotection in 20% sucrose and embedded in OCT. Eyes and brains were cryosectioned at 12 µm and retinal explants at 25 µm, and processed for staining on the same day. Slides were pre-incubated for 1 h in blocking buffer (1% BSA) and incubated overnight at 4°C with primary antibody (see supplementary Materials and Methods for details) diluted in blocking buffer. *In vivo* BrdU incorporation was done by intraperitoneal injection of BrdU (100 mg BrdU/kg) for 1 h. For detection of BrdU, sections were treated with 2 N HCl at 37°C for 30 min before incubation with primary antibodies. Primary antibodies were detected using appropriate secondary antibodies conjugated with Alexa Fluor 488, 555 or 594 (1:1000; Invitrogen) diluted in PBS at room temperature for 1 h. In all cases, nuclei were stained with Hoechst 33342 (1:20,000, Molecular Probes). The slides were mounted in Mowiol.

Retinal electroporation

DNA preparations were diluted in water containing 0.5% Fast Green and injected subretinally at P0 in pups. After the injection, tweezer-type electrodes (Gene paddles, 3 x 5 mm Paddles Model 542, Harvard Apparatus), soaked in PBS were placed on either side of the head over the eyes and five square pulses of 50 ms at 80 V were applied using a pulse generator (ECM 830 Square Wave Electroporation System, Harvard Apparatus). Pups were sacrificed after 24 h, the eyes were removed, fixed and processed for immunostaining as described above.

Quantitative analysis

The number of cells expressing the different cell type-specific markers was quantified by averaging the number of positive cells from three regions of 250 µm in the retina and brain. Statistical comparisons were done using two-tailed Student's *t*-test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.L. and M.C.; experimentation and data analysis: M.L., B.T., C.B.-P. and C.M.; manuscript writing: M.L. and M.C.; manuscript editing: B.T. and C.M.; supervision and funding: M.C.

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Supplementary information

Supplementary information available online at
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