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Abstract

In the vertebrate retina six types of neurons and one glial cell type are generated from multipotent retinal progenitor cells (RPCs) whose proliferation and differentiation are regulated by intrinsic and extrinsic factors. RPCs proliferate undergoing interkinetic nuclear migration within the neuroblastic layer, with their nuclei moving up and down along the apico-basal axis. Moreover, they only differentiate and therefore exit the cell cycle only at the apical side of the neuroblastic layer. Sema6A and its receptors PlexinA4 and PlexinA2 control lamina stratification of the inner plexiform layer in the mouse retina. Nevertheless, their function in earlier developmental stages are still unknown. Here, we analyzed the embryonic retina of PlexinA2 and Sema6A knock out mice. Using time-lapse videomicroscopy we provide evidence that Sema6A/PlexinA2 signaling participates to interkinetic nuclear migration of RPCs around birth. When disrupted, RPCs migration is blocked at the apical side of the neuroblastic layer. This is the first evidence supporting a role for transmembrane molecules in the regulation of interkinetic nuclear migration in the mouse retina.

Key words: interkinetic migration, retina, progenitor cell, PlexinA2, Sema6A.

Introduction

Visual information processing is contingent to a precise assembly of seven principal types of cells into the functional architecture of the retina. All these cells derive from multipotent retinal progenitor cells (RPCs) located in the neuroblastic layer (NBL) of the neuroretina (Marquardt 2003; Davis & Dyer 2010). Early during development, RPCs tend to divide symmetrically to increase the pool of progenitors. This is followed by differentiation and migration of retinal cells to their appropriate retinal layer. RPCs, as some other neural progenitors, exhibit interkinetic nuclear migration (INM), which is a back and forth nuclear movement along the apico-basal axis that is coordinated with the cell cycle advancement (reviewed in (Kosodo 2012; Spear & Erickson 2012). During the entire development, cell division in the retina takes place at the apical side, whether RPCs are in a proliferative or differentiating state. The cytoskeletal apparatus that directs retinal INM has mostly been studied in the zebrafish. Actomyosin is an essential driver of INM which is not impaired by disruption of the microtubules network (Norden *et al.* 2009). In the rodent neocortex it has also been shown that during INM, microtubules and actomyosin are required for nuclear positioning (Martini & Valdeolmillos 2010) and that nuclei migrate apically via dynein (Tsai *et al.* 2010). Interestingly, in the mouse retina, the non-muscle myosin IIB heavy chain is co-localized with dynein. It is then likely that rodent retinal INM proceeds with these same cytoskeleton molecules. Nuclear envelope proteins, such as SUN1 and SUN2 play a central role in INM in the retina to link the nucleus with dynein/dynactin and kinesin motor proteins (Yu et al. 2011). Mounting evidences support that

proliferation and cell fate specification of the RPCs are regulated by intrinsic factors (reviewed in (Ohsawa & Kageyama 2008; Agathocleous & Harris 2009), but also few extrinsic regulators (Anchan *et al.* 1991; Lillien & Cepko 1992; Wang *et al.* 2005; Martins & Pearson 2008). Nevertheless, no regulators of the INM itself have yet been identified.

The semaphorin family of guidance molecules comprises secreted and membrane-bound proteins that have major roles in different neuronal developmental processes, such as neuronal migration, dendritic arborization, axon guidance and branching (Pasterkamp & Giger 2009; Tran *et al.* 2009). Multiple classes of semaphorins are expressed in the developing mammalian retina (Leighton *et al.* 2001; de Winter *et al.* 2004).

Here, we investigated if PlexinA2 (Suto *et al.* 2007), one of the receptor of the transmembrane semaphoring Sem6A, is involved in the early development of the retina. In the nervous system, PlexinA2 mediates repulsive interactions between boundary cap cells and immature spinal motor neurons to regulate soma positioning (Bron *et al.* 2007). More interestingly, in the cerebellum, PlexinA2 interacts with Sema6A to control lamina-restricted projection of mossy fibers (Suto *et al.* 2007). In the retina, Sema6A-PlexinA2 signaling influences the stratifications of On and Off dendritic processes of starbust amacrine cells to different sublaminae of the inner plexiform layer (IPL) and controls the elaboration of the dendritic fields of symmetric On starbust amacrine cells . (Matsuoka *et al.* 2011; Sun *et al.* 2013). Sema6A, together with its other receptor, PlexinA4 are necessary for the formation of the outer plexiform layer (Matsuoka *et al.* 2012). Finally, Sema6A together with PlexinA2 and PlexinA4

are essential for the development of the accessory optic system (Sun *et al.* 2015).

Nevertheless, the function of PlexinA2 in early retinal development has not yet been addressed. In this study, we used *Sema6A*- and *PlexinA2*-deficient mice together with different markers of the different retinal cell types to investigate a possible role of PlexinA2 in the development of the retina.

We found that Sema6A and PlexinA2 are expressed in the NBL during retinal development and provide evidence that Sema6A-PlexinA2 signaling controls retinal INM.

Materials and Methods

Animals

C57Bl/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were used for *in situ* hybridization. *Plexin-A2* knockout mice and *Sema6A* knockout mice were previously described (Leighton *et al.* 2001; Suto *et al.* 2007) and were maintained on a C57Bl/6J background. The day of the vaginal plug was counted as embryonic day0 (E0) and the day of birth as postnatal day 0 (P0). All experimental protocols and procedures were approved by “Comité d'éthique en expérimentation animale n°5 (Charles Darwin)” from the Ministère de l’Education Nationale de l’Enseignement Supérieur et de la Recherche (authorization N°02339.02).

In situ hybridization

Antisense riboprobes were labeled with digoxigenin-11-D-UTP (Roche Diagnostics, Meylan, France) as described previously (Marillat *et al.* 2002), by *in vitro* transcription of mouse cDNAs encoding *PlexinA2* (Suto *et al.* 2007). Tissue sections were hybridized as described previously (Marillat *et al.* 2002). Sections were examined with a microscope (DM5000, Leica, Wetzlar, Germany) coupled to a CoolSnapFx camera (Roper Scientific, Lisses, France).

Immunocytochemistry

At embryonic stages, heads were fixed by overnight immersion in 4% paraformaldehyde (PFA) diluted in 0.12 M PB. Tissue was then cryoprotected in 10% sucrose and frozen as described in (Marillat *et al.* 2002). After birth, eyes were fixed 1 h by immersion in 4% PFA and cryoprotected in 30% sucrose. Sections were incubated with the following antibodies: Phosho-Histone H3 antibody (1/800; Cell Signaling Technology, Danvers, USA), Goat anti-PlexinA2 (1/400; Santa Cruz Biotechnology, USA), anti-mouse Sema6A (1/200, R&D systems, Abingdon, UK), anti-PAX6 (1/1000; Millipore, Darmstadt, Germany), rabbit polyclonal to Islet1 (1/1000; abcam ab20670, Cambridge, UK), anti-OTX2 (1/500, R&D systems), AP2ß antibody (1/150, Cell Signaling Technology). Sections were counterstained with Hoescht 33258 (10 mg/ml; Sigma, Saint-Louis, USA) mounted in Mowiol (Calbiochem, Darmstadt, Germany) and examined with a fluorescent confocal microscope (FV1000, Olympus, Tokyo, Japan). Cell counting was carried out on a computer screen in arbitrary length units in the central region of the retinal sections, and three sections from 3 different animals were counted.

EdU labeling

For EdU labeling at embryonic stages, pregnant mice were injected intraperitoneally with 100 mg EdU per kg body weight and sacrificed 2 h later. EdU detection was performed using the Click-iT EdU kit (Thermo Fisher Scientific, Waltham, USA).

DNA constructs

The pCX-EGFP plasmid (Niwa *et al.* 1991) was kindly provided by Dr Okabe (Osaka University) and prepared in endotoxin free condition (Qiagen, Hiden, Germany).

Retina electroporation

Heads were cut out from the E13-E14 embryos and 1 µl of pCX-EGFP diluted at a concentration 2.5 µg/µl in PBS (phosphate buffer saline) containing 0.2% (wt./vol.) fast green (Sigma) was injected into the eye using glass micropipettes. The head of each embryo was placed between tweezer-type electrodes (CUY650-P5 Nepagene, Chiba, Japan) four square electric pulses (40 V, 50 ms) were passed at 950 ms intervals using a CUY-21 electroporator (Nepagene). P0-P1 pups were anesthetized by chilling on ice, and a small incision was made in the eyelid and sclera near the lens with a 30-gauge needle. The same DNA solution as for embryonic retina was injected into the subretinal space through the incision by using a Hamilton syringe with a 32-gauge blunt-ended needle under a dissecting microscope.

Retina culture and time-lapse imaging

Following electroporation, embryonic heads were cut into 200 µm thick slices with a tissue chopper (McIlwain, Mickle Laboratory Engineering, Guildford, UK). Slices were transferred to a Millicell insert (Millipore, Darmstadt, Germany) and cultured for 24-48 h to 5 days in F12:DMEM (Gibco, Thermo Fisher Scientific), glucose 0.5%, L-glutamine 2 mM, BSA 0.05%, penicillin/streptomycin 100 U/ml, and ITS supplement 1% (insulin/transferin/selenite; Sigma). For P0 retina, the whole retina was folded and placed on the millicell. For time-lapse imaging, slices were embedded in collagen gel, soaked in culture medium and transferred on the insert to a 37°C stage incubator chamber (Life imaging Service, Switzerland) adapted to an upright microscope (DM6000 Leica) and provided with constant gas flow (5% CO2, 10% air). Slices were allowed to settle for 1 h and then were imaged with a spinning disk confocal system with a 491 nm excitation filter and a Coolsnap HQ2 CCD camera (all from Roper scientific). Z stacks ($\Delta z=3$) were obtained every 30 min using a 40X water-immersion objective (Leica, NA 0.5). Image processing was carried out using Image J.

Statistical analysis

Statistical analyses were performed in Prism 6 (GraphPad Software, San Diego, USA). All data are reported as mean ± SEM. The statistical significance was tested by a multiple t-test with the Holm-Sidak method. Significance was set at $p<0.05$.

Results

The temporal and spatial patterns of PlexinA2 expression suggest a role in retinal development

The PlexinA2 receptor is known to regulate dendritic morphology of On-starbust amacrine cells (Sun *et al.* 2013) in the postnatal retina, but its role at earlier stages has not been studied. To address this question, we first studied PlexinA2 expression pattern in the developing retina. We detected faint *PlexinA2* mRNA expression at embryonic day 14 (E14, n = 3) (Fig. 1A), concentrated in the central part of the ganglion cell layer (GCL) of the retina, but also in the outermost part of the neuroblastic layer (NBL). A similar expression pattern was observed for *Sema6A* mRNA (Fig. 1B; n = 3). To confirm that PlexinA2 and Sema6A were expressed in embryonic retina, we immunostained E14 coronal sections and found the same expression pattern (Fig 1C, D; n = 3). The specificity of the PlexinA2 antibody was confirmed by the absence of staining on retina sections from E13, P4 and adult *PlexinA2*$^{-/-}$ mice (Fig 1E and not shown). At E16, the IPL starts forming and was strongly labeled by the PlexinA2 antibody (Fig 1F; n=3). Double labeling for PlexinA2 and Islet1 revealed a colocalisation in retinal ganglion cells (RGCs) and amacrine cells. In the NBL, PlexinA2 was detected in numerous RPCs. After birth, a strong PlexinA2 immunolabeling was still found in the IPL (Fig 1G,H; P1 n = 3, P3 n = 3). In the NBL, PlexinA2 was mainly expressed at the apical part, where the RPCs are at the G2/M cell cycle phase, as shown by the expression of H3P (a marker of mitotic cells). About 54.8 ± 8.0% of H3P-positive cells are also

PlexinA2-positive. At P3, in the NBL PlexinA2 was co-localized with Pax6, a master regulatory gene essential for early retinal specification that controls the expression of transcription factors which direct neurogenesis (Hill *et al.* 1991; Marquardt *et al.* 2001). Interestingly, almost all the Pax6-positive cell of the NBP colocalized with Pax2 (95.5 ± 3.1%) confirming that PlexinA2 was still expressed by RPCs. Thus, it appears that RPCs express PlexinA2 and in particular at the apical part of the NBL where they are at the G2/M phase, suggesting that Sema6A/PlexinA2 signaling might control retinal cell proliferation, differentiation and migration.

The absence of PlexinA2 or Sema6A does not affect RPCs proliferation

PlexinA1 has been recently shown to alter the proliferative ability of neuronal progenitors in the developing cortex (Andrews *et al.* 2016). To determine if PlexinA2 and Sema6A could influence RPCs proliferation, E13, E14 and E18 (n = 3 cases for each) *PlexinA2*- and *Sema6A*-knockout mice were pulse labeled for 2 hours with 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analogue that becomes incorporated into DNA during S phase of the cell cycle (Fig 2A,B). RPCs proliferate and differentiate at different rates whether they are situated at the peripheral or the central part of the retina (Young 1985). Therefore, the retina was divided into four zones (Fig 2C) and the number of EdU-positive cells and the total number of cells counted in each bin (n = 3). We found that the number of EdU-positive cells in the retina was similar in *PlexinA2*$^{-/-}$ or *Sema6A*$^{-/-}$ mutant mice compared with control littermates. At E13, EdU-positive cells represented around 40% of total cell number in the four zones (Fig 2D). For

instance, in zone 4, we counted 36.83 ± 1.20 % of EdU^+ cells in wild-type mice, 37.12 ± 4.83 % of EdU^+ cells in *PlexinA2*$^{-/-}$ ($p<1$), and 36.21 ± 1.15 % of EdU^+ cells in Sema6A$^{-/-}$ ($p<0.8$). One day later, the number of EdU-positive cells was slightly higher compared to E13, but there was still no significant difference between retinas of wild-type and PlexinA2 or Sema6A mutants (Fig 2E; zone 4, wild type 46.19 ± 2.76 % of EdU^+ cells, PlexinA2$^{-/-}$ 36.79 ± 4.05 % of EdU^+ cells, $p<0.2$, Sema6A$^{-/-}$ 40.70 ± 1.42 % EdU^+ cells, $p<0.2$; n = 3 independent experiments). Finally, at E18 we found that the proportion of EdU-positive cells was lower (less than 30%) compared to E13-E14 but without any significant differences between the different genotype (Fig 2F; zone 4 wild type 27.02 ± 0.276 % of EdU^+ cells; PlexinA2$^{-/-}$ 27.35 ± 0.53 % of EdU^+ cells, $p<0.7$; Sema6A$^{-/-}$ 24.67 ± 2.01 % EdU^+ cells, $p<0.7$; n = 3 independent experiments). These results show that the lack of PlexinA2 or Sema6A does not alter the proliferation of RPCs.

Cell differentiation in PlexinA2 and Sema6A KO retinas

To investigate the cell fate determination and differentiation in the retina, we performed immunohistochemistry of E13, E14, and E18 retinas (n = 3 for each case), using Islet1 a marker of RGC, bipolar and cholinergic amacrine cells (Elshatory *et al.* 2007). There was no significant difference in cell fate determination and differentiation at E13 in any zone counted (Fig 3C). Nevertheless, we found a significantly higher proportion of Islet1-positive cells in the NBL in the mutant retina when we quantified the whole retina: 24.88 ± 1.11% *vs* 34.54 ± 3.40% $Islet1^+$ cells in *PlexinA2* KO ($p<0.02$) and

34.73 ± 2.64% Islet1^{+} cells in *Sema6A* KO ($p<0.004$; Fig 3C). Just a day later, we found that in zone 2 and 4, the amount of Islet1-positive cells in the NBL was significantly increased in *PlexinA2* KO compared to wild-type (zone 2, 22.87 ± 1.46% *vs* 45.58 ± 4.40% Islet1^{+} cells in the NBL, $p = 0.01$; zone 4, 26.81 ±1.65 *vs* 54.47 ± 2.90 Islet1^{+} cells in the NBL, $p = 0.001$; $n = 3$ independent experiments), but not in Sema6A$^{-/-}$ retina (zone 2, 34.29 ± 3.27% Islet1^{+} cells in the NBL, $p = 0.26$; zone 4, 20.15 ± 2.50% Islet1^{+} cells in the NBL, $p = 0.09$; $n = 3$ independent experiments; Fig 3 A,B,D). At E18, the number of Islet-positive cells in the NBL was considerably decreased regardless of the zone (less than 10 Islet1^{+} cells in wild-type retinas, and without any significant difference in the *PlexinA2*- or *Sema6A*-deficient mice (Fig 3E).

Deletion of PlexinA2 and Sema6A leads to a migration defect

Sema6A-PlexinA2 signaling modulates granule cell migration in the cerebellum (Renaud *et al.* 2008). Therefore, we decided to investigate whether these molecules could also be involved in RPCs migration. In order to visualize migrating RPCs, retinas were electroporated with pCX-GFP, an expression vector encoding green fluorescent protein (GFP) previously used to study the neuronal migration by time-lapse videomicroscopy (Watanabe & Murakami 2009 ; Renaud & Chédotal 2014). GFP^{+} cells were first detected 20 h after electroporation. The retina was then imaged using spinning-disk time-lapse microscopy. This allowed us to follow several GFP+ cells located in the NBL. In control P0-P1 retinas (n = 16 retinas, fourteen independent experiments, 30 min interval over 20-30 h), GFP+ cells exhibited elongated bipolar morphologies as

they migrated (Fig. 4A). Nuclear movement toward the apical side was saltatory, as described for cortical interneurons and radially migrating granule cells (Fig. 4A; 21/23 cells). The RPC cell body suddenly jumped to the apical side of the NBL and stayed about 1.38 ± 0.6 hours at the apical side before a daughter cell appeared and started migrating to the basal side (21/21 cells observed; supporting movie S1). In contrast to controls, PlexinA2 KO RPCs did not migrate back to the basal side after reaching the apical side, for at least 10.4 ± 4.6 h (9/9 cells observed, five independent experiments, 30 min interval over 20-30 h; supporting movie S2). Interestingly, a comparable delay was observed at the G2/M phase in retina slices from *Sema6A*-deficient mice: RPCs stayed 8.09 ± 3.2 hours at the apical side of the NBL (5/5 cells observed three independent experiments, 30 min interval over 20-30 h). In almost all cases, it was not possible to determine if the RPC that had reached the apical side of the NBL couldn't proceed to mitosis or if they divided but that the daughter cells were not able to migrate. The latter seemed to have occurred in at least one Sema6A$^{-/-}$ retinal slice (Fig 4D): the RPC reached the apical side and stayed there for about 2 h before undergoing mitosis but the two daughter cells remained at the apical side for at least 5 h. These results suggest that PlexinA2/Sema6A signaling could control the interkinetic migration of RPCs.

RPCs are delayed at the G2/M phase in *PlexinA2*- and *Sema6A*-deficient mice

RPCs at the apical side of the NBL have reached the G2/M phase and expressed phospho-histone H3 (H3P). Therefore, we immunostained retinal

sections of *PlexinA2* and *Sema6A* knockout mice at different stages and quantified the number of H3P positive cells (Fig 5A,B). At E13 and E14 (n = 3 independent experiments for each), in each retinal zone, a similar proportion of H3P-positive cells (around 2 % of the total cell number of each zone) was found for all genotypes (Fig 5C,D). In contrast, at E18 (n = 3 independent experiments), the number of H3P-positive cells was always significantly higher in each of the four retinal zones, in both PlexinA2 and Sema6A knockout mice (Fig 5E). For instance in zone 4, we counted 0.54 ± 0.025 % of $H3P^+$ cells, and 1.18 ± 0.11% in *PlexinA2*$^{-/-}$ mice ($p<0.0001$) and 1.96 ± 0.29% $H3P^+$ cells in *Sema6A*$^{-/-}$ mice ($p<0.0001$). Therefore, the number of RPCs at G2/M phase was increased at E18.

Discussion

In this study, we found that during retinal development, PlexinA2 and Sema6A are expressed in RPCs and that perturbation of their expression around birth, maintains RPCs at the G2/M phase at the apical side of the NBL. Together, these results suggest that PlexinA2 and Sema6A deletion perturbs the migration of RPCs without affecting their proliferative activity. Therefore, the altered migration is, probably, caused by a direct activity of PlexinA2 and Sema6A on retinal cell fate decision, rather than secondary to a perturbation of the timing of cell cycle exit. To our knowledge, this is the first time, that membrane bound molecules are implicated in the migration of RPCs, in particular in the mouse. Nevertheless, we were unable to determine whether the division of RPCs was delayed after they reached the apical side of if cell

division occurred normally, but that the two daughter cells were unable to migrate to the basal side. However, in one case of Sema6A$^{-/-}$, we could observe that two daughter cells remained together at the apical side for 5 h (Fig 4C). Unfortunately, we were not able to record the cells long enough and therefore cannot say whether the two daughter cells were definitively blocked at the apical side or if they finally migrate toward the basal side.

Most of the time RPCs are pausing, just moving a little at their position, and at some point proceed to INM. It is therefore very difficult to catch an RPC undergoing INM and we had to make more than 100 movies for this study.

The fate of blocked RPCs will need to be investigated further. Their migration toward the basal side could only be delayed as supported by the lack of obvious retina cell layering defect in PlexinA2 or Sema6A knockout mice besides the IPL disruption (Matsuoka *et al.* 2011). Interestingly, the development of retinal vessels was also, reported to be delayed in *Sema6A*-deficient mice although it ultimately achieves a full development (Segarra et al. 2012). Moreover, we showed that this migration defect occurs mainly around birth and therefore that it probably does not affect all RPCs.

In the cerebellum, it has already been shown that granule cells fail to migrate in absence of PlexinA2 (Renaud *et al.* 2008). In addition, granule cell migration defects in both *PlexinA2* and *Sema6A*-deficient mice are age-dependent (Renaud & Chédotal 2014). Finally, as in the retina, the proliferation of granule cell progenitors is not affected (Renaud *et al.* 2008). The time lapse videomicroscopy experiments clearly suggest that cerebellar migration defects are probably due to a failure of nuclear translocation. Indeed, granule cells

tangential migration proceeds by nucleokinesis comparably to interkinetic migration of RPCs. Furthermore, in cerebellar granule cells, microtubules linked to the centrosome appear to pull the nucleus forward during migration and an abnormal coupling of the centrosome-and nucleus uncoupling was detected in *Sema6A*- and *PlexinA2*-deficient mice. *In vitro* experiments using EGL explants showed that the distance between the centrosome and the nucleus in *Sema6A*$^{-/-}$ and *PlexinA2*$^{-/-}$ mice were affected (Renaud et al., 2008). Nevertheless, in the zebrafish retina the apical localization of the centrosome is not a precondition for nuclear migration, as apical INM still happens when centrosomes and nuclei meet non-apically or in cases when centrosome integrity is perturbed (Strzyz *et al.* 2015).

During retinal neurogenesis, at the apical side Notch/Delta signaling inhibits neighboring RPCs from differentiating prematurely in order to maintain the RPC pool (Jadhav *et al.* 2006; Yaron *et al.* 2006 ; Del Bene *et al.* 2008 ; Rocha *et al.* 2009). For instance, Dll4 (a Notch ligand expressed in RPCs) initiates RPC proliferation but mainly blocks photoreceptor differentiation (Luo *et al.* 2012). An interaction between Sema/Plexin and Notch signaling has already been observed: during developmental angiogenesis Sema3E/PlexinD1 disrupts Notch activity (Kim *et al.* 2011). Therefore another possible hypothesis is that the Sema6A/PlexinA2 could interact with Notch signaling. A knock down of Sema6A/PlexinA2 function could delay RPCs to proceed to differentiation or to stay in cycle as a progenitor cell.

To date, most of the molecules known to control retinal development are transcription factors, but their target genes, in particular encoding molecules

mediating cell-cell interactions, are still largely unknown. Here our findings reveal that membrane bound molecules, Sema6A/PlexinA2 are implicated in the migration of RPCs. Future studies should unravel new cell interactions molecules to complete the understanding of RPCs migration.

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Figure Legends

Figure 1. Spatio-temporal expression pattern of PlexinA2 and Sema6A in the developing retina. *In situ* hybridization for *PlexinA2* mRNA (A) and *Sema6A* (B) on coronal sections of E14 mouse eye. PlexinA2 immunocytochemistry at E14 (C) E16 (F), P1 (G) and P3 (H). (D) Sema6A immunocytochemistry on E14 retina coronal section. (A-D) At E14, a low expression of *PlexinA2* and *Sema6A* mRNAs (A, B) and proteins (C, C) expression is detected in the neuroblastic layer (NBL) and the ganglion cell layer (GCL). (E) The PlexinA2 antibody does not show any labeling in the retina of the *PlexinA2*-deficient mice. At 16, PlexinA2 is expressed in NBL and the IPL. (F) At E16 double labeling with Islet1 (magenta) shows that, besides its expression in the RPCs (arrowheads), PlexinA2 (green) is expressed in RGCs, newborn amacrine cells and the nascent IPL. (G) Later at P1, PlexinA2 expression in the NBL is mostly at the apical side where the RPCs are at the G2/M phase (H3P cells, magenta) some of the H3P+ cells are also PlexinA2+ (arrowheads). (H) At P3 PlexinA2 is still detected at the apical side of the NBL and co-expressed with Pax6 (magenta) in RPCs (arrowheads). Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; NBL, neuroblastic layer. Scale bars: A-F, 100 µm; G and H, 25 µm.

Figure 2. Proliferation is not affected in *PlexinA2*- and *Sema6A*-deficient mice embryonic retina. (A, B) E18 retina coronal sections showing EdU labeling (green) and DAPI (white). (C) Half of the retina was divided in four bins (zone1 to 4) and (D-F) the number of EdU positive cells counted as well as the total cell

number (DAPI). At E13(C), E14 (D) and E18 (E) the percentage of EdU+ cells is not significantly different between *wild-type* and *PlexinA2*- or *Sema6A*-deficient retina. Abbreviations: GCL, ganglion cell layer; NBL, neuroblastic layer. Error bars indicate SEM. Scale bars: 25 µm.

Figure 3. Specification of RGC and bipolar cell precursor is perturbed in *PlexinA2*-deficient mice embryonic retina. (A, B, F, G, I, J) E14 retina coronal sections counterstained with DAPI (white) labeled with antibodies against Islet1 (green in A, B), OTX2 (green in F, G) and AP2ß (green in I, J). (C-E, H, K) Half of the retina was divided in four bins (zones 1 to 4) and the number of Islet1-positive cells and the total number of $Isl1^+$cells were counted (C-E). At E13(C) and E14 (D) the percentage of Islet1+ cells in the NBL of *PlexinA2*- or *Sema6A*-deficient retina is significantly higher compared to wild-type retina but not at E18 (E). Number of $OTX2^+$ cells (cone photoreceptor precursors) is not affected in *PlexinA2*- or *Sema6A*-deficient retina at E14 (H). Quantification of $AP2ß^+$ cells in E14 retina showing that amacrine cell differentiation is not affected in *PlexinA2*- or *Sema6A*-deficient mice (K). Abbreviations: GCL, ganglion cell layer; NBL, neuroblastic layer. Error bars indicate SEM. * $p>0.05$, ** $p<0.01$, *** $p<0.001$. Scale bars: 25 µm.

Figure 4. RPC daughter cells fail to migrate in *PlexinA2* and Sema6A mutants

Time lapse video-microscopy on P0 retina of wild-type (A) and PlexinA2KO (B) mice. (A) An RPC cell, labeled with GFP migrates to the apical side of the NBL and divides into two daughter cells. (B) In *PlexinA2*-deficient mice, RPC

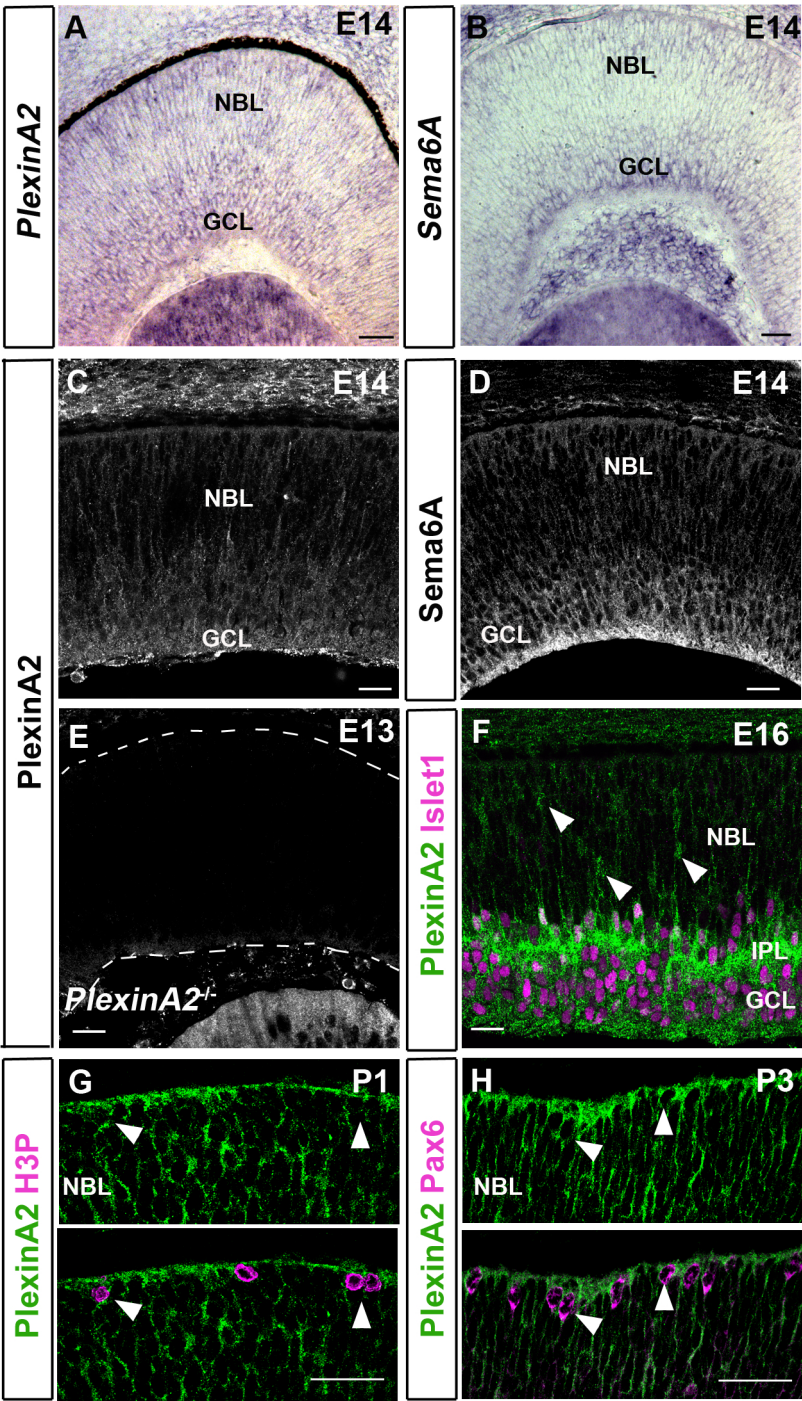
reaches the apical side but the daughter cell fail to migrate to the basal side. (C) In *Sema6A*-deficient mice, RPC reaches the apical side, divide in two daughter cells which stay at the apical side for 5 h. Time in hr:min. Scale bars represent 10 μm.

Figure 5. Increased number of H3P+ cells in *PlexinA2*$^{-/-}$ and *Sema6A*$^{-/-}$ mice. Coronal retina sections from wild-type (A) and *PlexinA2*$^{-/-}$ (B) at E18 were immunostained with H3P (arrowheads). Quantification of $H3P^{+}$ cells at E13 (C), E14 (D) and E18 (E). Error bars indicate SEM (*** $p<0.001$). Abbreviations: NBL, neuroblastic layer. Error bars indicate SEM. Scale bars: 25 μm.

Supplementary Movie 1: Time-lapse movie (510 min) corresponding to Fig 4a, of a wild-type RPC migrating to the apical side and then dividing in two daughter cells in a P0 retina image 24h after electroporation. The apical side in on the top of the frame.

Supplementary Movie 2: Time-lapse movie (1470 min) corresponding to Fig 4b, of a PlexinA2$^{-/-}$ RPC migrating to the apical side but the daughter cells fail to migrate to the basal side in a P0 retina image 24h after electroporation. The apical side in on the top of the frame.

Figure 1


PlexinA2
A
E14
NBL
GCL
Sema6A
B
E14
NBL
GCL
PlexinA2
C
E14
NBL
GCL
Sema6A
D
E14
NBL
GCL
E
E13
PlexinA2-/-
PlexinA2 Islet1
F
E16
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IPL
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G
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NBL
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H
P3
NBL

Figure 2

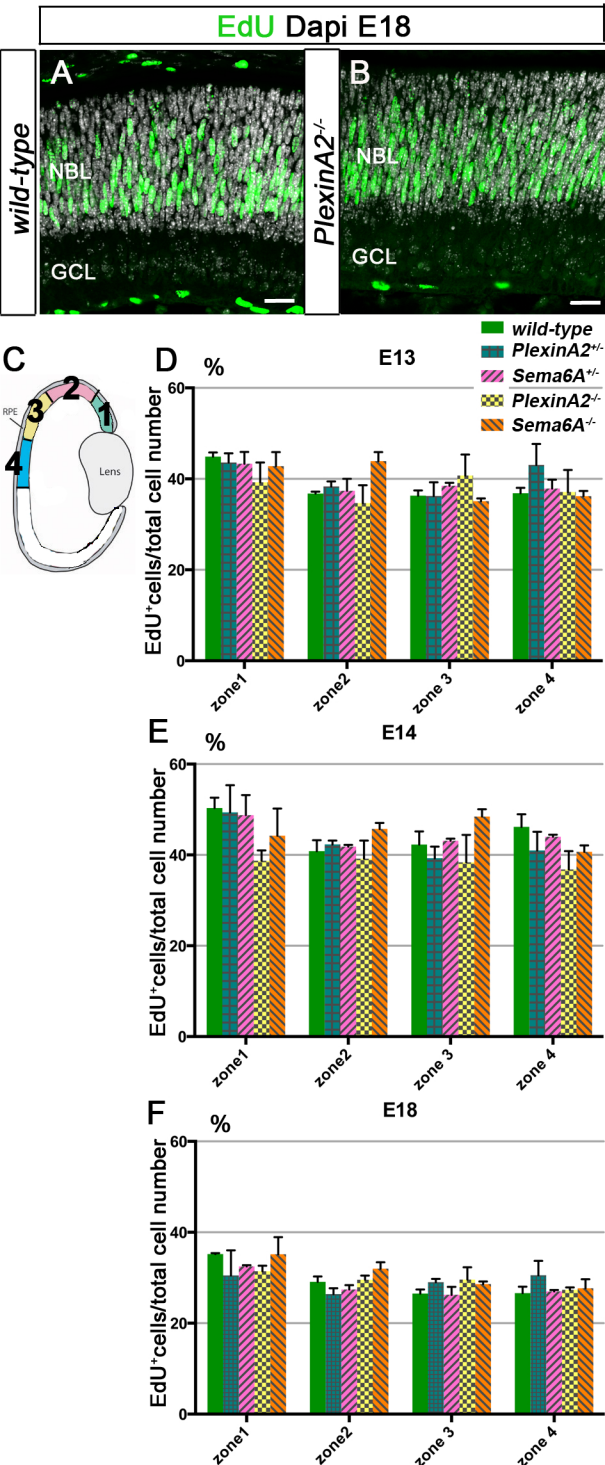
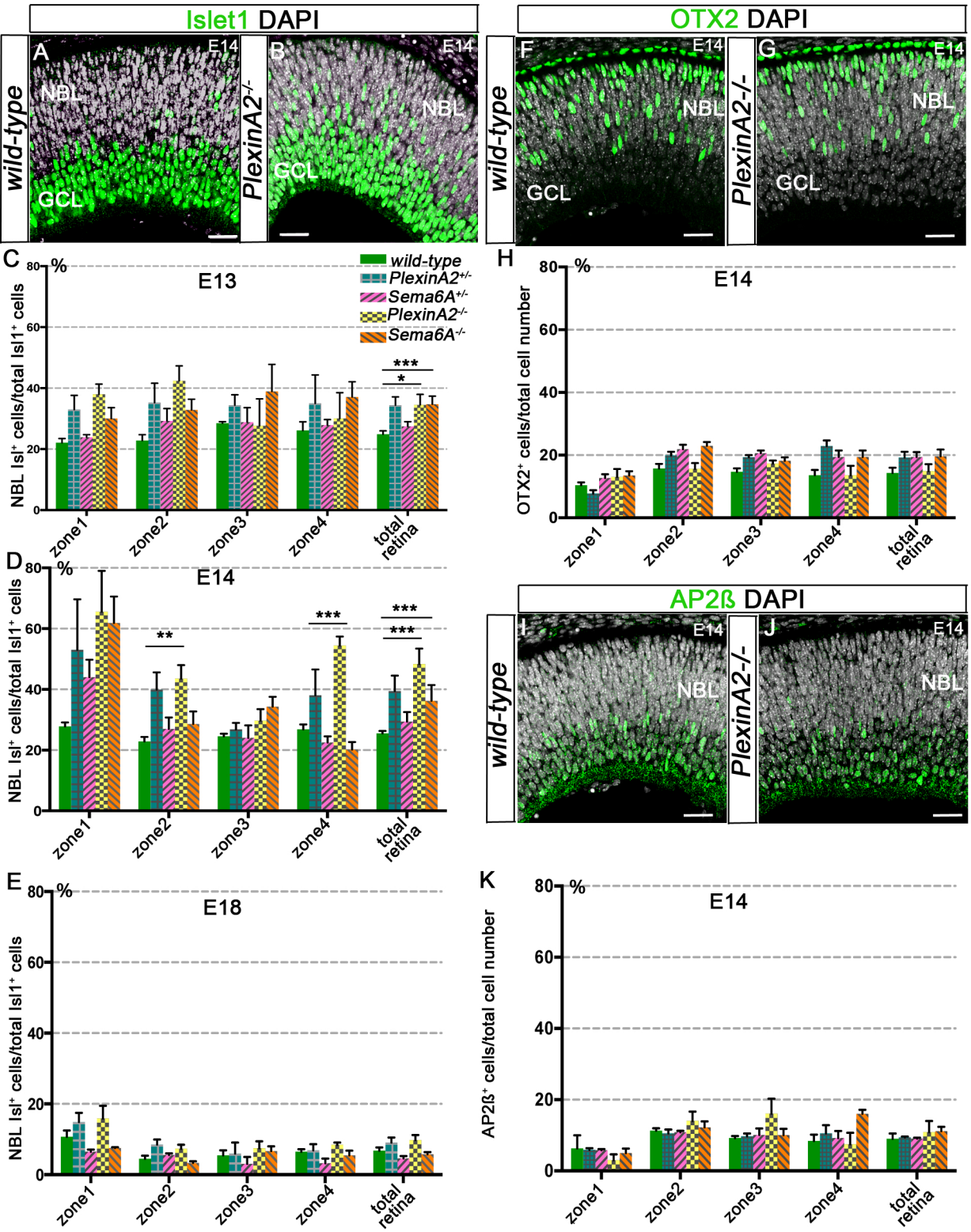

EdU Dapi E18
wild-type
PlexinA2-/-
A
B
NBL
GCL
NBL
GCL
C
RPE
1
2
3
4
Lens
D
%
E13
wild-type
PlexinA2+/-
Sema6A+/-
PlexinA2-/-
Sema6A-/-
EdU+cells/total cell number
60
40
20
0
zone1
zone2
zone 3
zone 4
E
%
E14
EdU+cells/total cell number
60
40
20
0
zone1
zone2
zone 3
zone 4
F
%
E18
EdU+cells/total cell number
60
40
20
0
zone1
zone2
zone 3
zone 4

Figure 3


Islet1 DAPI
A
E14
NBL
GCL
wild-type
B
E14
NBL
GCL
PlexinA2-/-
OTX2 DAPI
F
E14
NBL
GCL
wild-type
G
E14
NBL
GCL
PlexinA2-/-
C
%
E13
wild-type
PlexinA2+/-
Sema6A+/-
PlexinA2-/-
Sema6A-/-
NBL Isl+ cells/total Isl1+ cells
***
*
zone1
zone2
zone3
zone4
total retina
H
%
E14
OTX2+ cells/total cell number
zone1
zone2
zone3
zone4
total retina
D
%
E14
NBL Isl+ cells/total Isl1+ cells
**
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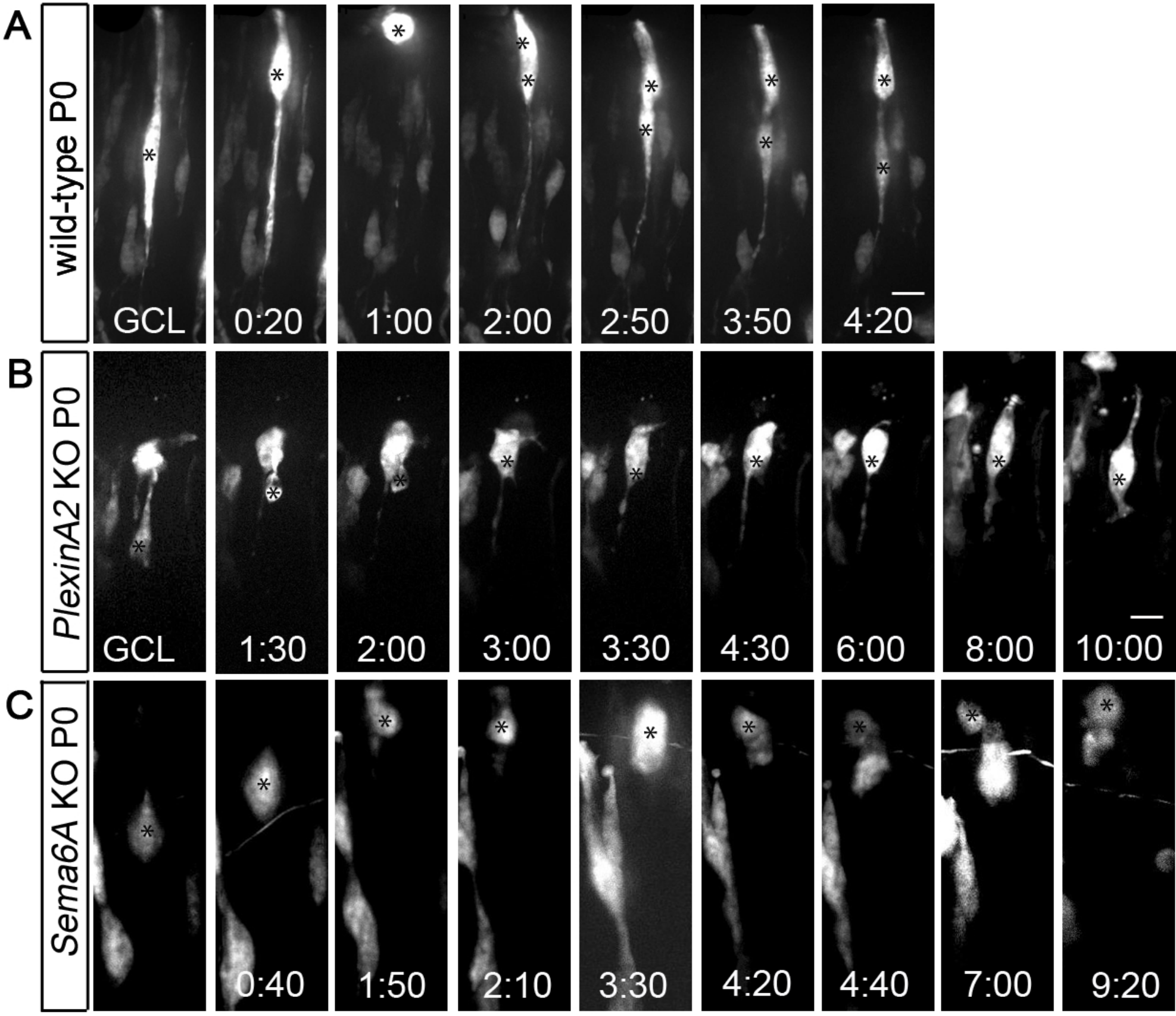
***
zone1
zone2
zone3
zone4
total retina
AP2ß DAPI
I
E14
NBL
wild-type
J
E14
NBL
PlexinA2-/-
E
%
E18
NBL Isl+ cells/total Isl1+ cells
zone1
zone2
zone3
zone4
total retina
K
%
E14
AP2ß+ cells/total cell number
zone1
zone2
zone3
zone4
total retina

Figure 4


A
wild-type P0
GCL
0:20
1:00
2:00
2:50
3:50
4:20
B
PlexinA2 KO P0
GCL
1:30
2:00
3:00
3:30
4:30
6:00
8:00
10:00
C
Sema6A KO P0
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1:50
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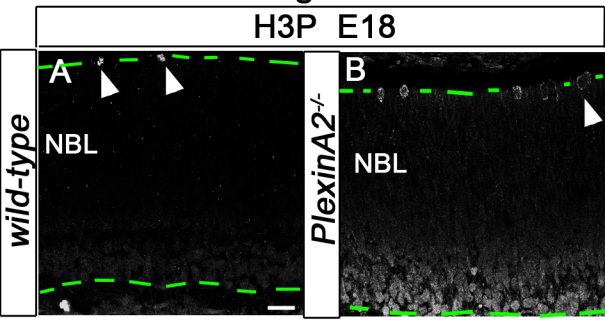
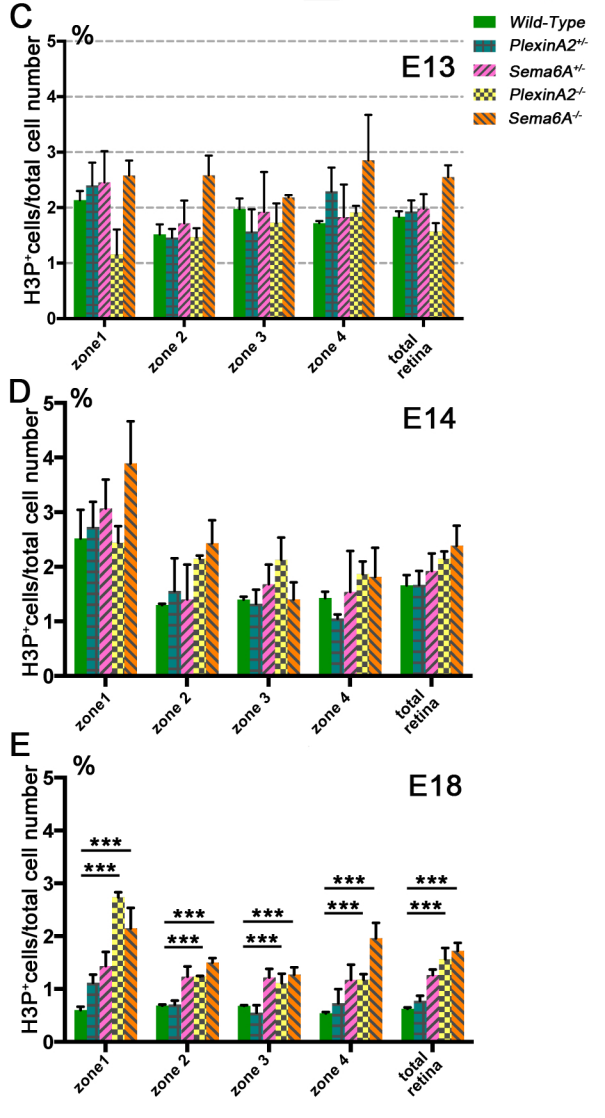
3:30
4:20
4:40
7:00
9:20

Figure 5


H3P E18
wild-type
PlexinA2-/-
A
B
NBL
NBL


C
D
E
%
E13
E14
E18
Wild-Type
PlexinA2+/-
Sema6A+/-
PlexinA2-/-
Sema6A-/-
H3P+cells/total cell number
zone1
zone 2
zone 3
zone 4
total retina
***