The Role of Glial Cells During Differentiation of Mouse Retinal Organoids

Die Rolle von Gliazellen bei der Differenzierung von retinalen Organoiden

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Eidesstattliche Erklärung

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Abstract

The retina is the sensory organ required for the collection of visual stimuli. It consists of six basic retinal neurons and one supporting glia cell type. Rod and cone photoreceptors detect incoming photons and translate the information into electrochemical cues. Interneurons include bipolar cells, amacrine cells, horizontal cells, and ganglion cells. They perform first processing in the retinal neuronal network and transmit the information inside the retina or to the brain via the optic nerve.

To facilitate the study of retinal development and disease progression in an *in vitro* model, organoids were established, which reduced some of the drawbacks of animal models. Retinal organoids (RO) resemble the retina in many ways and recapitulate the early developmental processes involved in retinogenesis. Similarities include the generation of all retinal cell types from multipotent progenitors as well as the layering of the cells according to their *in vivo* positioning. Nevertheless, the cultivation time of ROs is limited, the layering lacks complexity, and Müller glia remain immature and in low numbers. Other support cells, like retinal astrocytes and microglia, are completely absent. Retinal glia cells are dynamic and active participants in the retina's response to physiological and pathological stimuli, highlighting their importance in the overall sensory function of the eye. Since the differentiation of ROs relies on parameters that mimic their *in vivo* development, co-cultivation of retinal neurons with supporting glia cells was targeted in this work.

First, the protocol for the generation of ROs was established. The ROs were characterised with regard to the cell numbers and distribution of neurons in the tissue and to confirm the possible absence of glia cells. To substitute for the lack of support cells, experiments were designed that did not disrupt organoid integrity but enabled glia-mediated support. This included targeting intercellular signalling via Notch ligands to increase the portion of glia cells in the developing ROs. In parallel, the procedure to isolate cortical astrocytes from mice was established. They were used for co-cultivation approaches combining a confluent monolayer of primary astrocytes with free-floating ROs. The culture medium was kept in favour of the ROs, and even though the astrocyte morphology was not altered, negative effects on their secretion behaviour could not be excluded. Thus, conditioned medium from astrocyte cultures was collected to treat ROs with potential secreted factors. In the co-cultivation experiments conducted, changes in neuron numbers or their apico-basal layering were not observed. The astrocyte conditioned medium did not improve the culture conditions and alterations were rather allocated to variations between the culture medium of ROs and astrocytes.

To enable cell-cell contact-mediated signalling between the retinal cells and the astrocytes, the ROs were dissociated, and reaggregated together with astrocytes. Reaggregated ROs showed similarities to the regular cultured ROs in regard to their cellular composition and organisation, as shown by immunocytochemical staining. The integration of astrocytes into reaggregated ROs, however, resulted in the loss of neuroepithelial organisation. To counter this loss of structure, an artificial basal side was introduced by the integration of a “glia core”, mimicking the retinal astrocytes residing in the ganglion cell layer *in vivo*. Providing basal cues to facilitate the reorganisation of retinal cells did not rescue the epithelial structure, and it was therefore concluded that the cell type was not suited for the perused goal.

In summary, co-cultivation approaches targeting multiple mechanisms of action were established but could not provide beneficial environmental cues that improved RO cultures. In the course of these experiments, the established method for the reaggregation of ROs is nevertheless a promising model to investigate neuroepithelial reorganisation and could pave the way for further co-cultivation studies with potentially advantageous cell types.

Zusammenfassung

Die Retina ist das Sinnesorgan, welches für die Aufnahme visueller Reize verantwortlich ist. Sie besteht aus sechs retinalen Neuronen und einem Glia-Zelltyp. Stäbchen- und Zapfen-Photorezeptoren detektieren einfallende Photonen und wandeln die Information in elektrochemische Signale um. Interneuronen umfassen bipolare Zellen, amakrine Zellen, horizontale Zellen und Ganglienzellen. Sie übernehmen die erste Verarbeitung der Informationen in der Retina und leiten diese anschließend innerhalb der Retina oder über den Sehnerv an das Gehirn weiter.

Um die Entwicklung der Retina und deren Erkrankungen in einem *In vitro*-Modell erforschen zu können, wurden retinale Organoide (RO) entwickelt. Diese stellen eine Alternative zu Tiermodellen dar. RO ähneln der Retina in vielerlei Hinsicht und bilden die frühen Entwicklungsprozesse der Retinogenese nach. Dazu gehört die Entstehung aller retinalen Zelltypen aus multipotenten Vorläuferzellen sowie die Schichtung der Zellen entsprechend ihrer Position in der Retina. Die Kultivierungsdauer von RO ist jedoch begrenzt, die Schichtung weist eine mangelnde Komplexität auf und Müller-Gliazellen bleiben unreif und in geringer Anzahl. Andere Glia-Zellen, wie retinale Astrozyten und Mikroglia, fehlen vollständig. Glia-Zellen sind dynamische und aktive Bestandteile der Retina und reagieren auf physiologische und pathologische Reize, was ihre Bedeutung für die Funktion des Auges unterstreicht. Da die Differenzierung von RO auf Parametern beruht, die ihre Entwicklung *in vivo* nachahmen, zielte diese Arbeit auf die Co-Kultur retinaler Neuronen mit Glia-Zellen ab.

Zunächst wurde das Protokoll zur Herstellung von RO etabliert. Die RO wurden hinsichtlich der Zellzahlen und der apico-basalen Verteilung der Neuronen im Epithel charakterisiert und die Abwesenheit von Müller-Glia bestätigt. Um das Fehlen der Glia-Zellen auszugleichen, wurden Experimente entworfen, die die Integrität der Organoide nicht beeinträchtigen, aber die Unterstützung durch Glia-Zellen ermöglichen. Dazu gehörte die Aktivierung des Notch-Signalwegs, um den Anteil von differenzierenden Glia-Zellen in den RO zu erhöhen. Parallel dazu wurde ein Protokoll zur Isolation kortikaler Astrozyten aus Mäusen etabliert. Diese wurden für Co-Kultur-Ansätze verwendet, bei denen primäre Astrozyten mit freischwimmenden RO kombiniert wurden. Das Kulturmedium wurde zugunsten der RO gewählt, weshalb negative Auswirkungen auf das Sekretionsverhalten der Astrozyten nicht ausgeschlossen werden konnten. Darum wurden die RO zusätzlich mit konditioniertem Medium behandelt. Die Co-Kultur führte zu keiner Veränderung der Anzahl oder der apico-basalen Schichtung der Neuronen. Durch das konditionierte Medium verursachte Veränderungen wurden auf Unterschiede zwischen den Kulturmedien der RO und der Astrozyten zurückgeführt.

Um Zell-Zell-Kontakte zwischen den retinalen Zellen und den Astrozyten zu ermöglichen, wurden die RO dissoziiert und zusammen mit Astrozyten reaggregiert. Reaggregierte RO zeigten in Bezug auf ihre Zusammensetzung und Schichtung Ähnlichkeiten zu den regulären RO, wie durch Immunfärbungen nachgewiesen wurde. Die Integration von Astrozyten in reaggregierte RO führte jedoch zum Verlust der epithelialen Organisation. Um dem entgegenzuwirken, wurde eine künstliche basale Seite durch die Integration eines „Gliakernes“ eingeführt, welcher die basalen retinalen Astrozyten der Retina nachahmt. Die Bereitstellung von basalen Signalen konnte die epitheliale Struktur jedoch nicht wiederherstellen, weshalb die Astrozyten eventuell für die Integration nicht geeignet sind.

Zusammenfassend wurden Co-Kultur-Ansätze mit unterschiedlichen Wirkungsmechanismen etabliert, die aber keinen positiven Effekt auf die Kultivierung retinaler Zellen zeigten. Die im Verlauf dieser Experimente etablierte Methode zur Herstellung von reaggregierten RO ist dennoch ein vielversprechendes Modell zur Untersuchung der neuroepithelialen Organisation.

Introduction

Among all senses, humans and most animals rely primarily on vision. The navigation through three-dimensional surroundings by the perception of their environment requires the interplay of several specialised tissues. Incidental light gets focused into the eye by the transparent dome-shaped cornea. Thereafter, light passes the anterior chamber and falls through the pupil onto the lens. The pupil is surrounded by the iris, which can constrict its opening to modulate the amount of infalling light by muscle contraction or relaxation. In the lens, light gets focused onto the retina, which lies on the back of the vitreous body (Graw 2010). In the retina, six major neuronal cell types form a neuronal circuit that enables the detection of visual cues, does first processing and transmits the signals for further processing to the brain (T. Harada, Harada, and Parada 2007). As a part of the central nervous system that is accessible and almost entirely built by a common progenitor pool, the retina is heavily studied and serves as a model for neurogenesis. Besides its utility in basic research, retinal tissue is a subject of medical research. With an increasingly aging population, diseases like age related macular degeneration or glaucoma, which can lead to visual impairment or even blindness, have become more prevalent (Evangelho, Mastronardi, and de-la-Torre 2019; Soundara Pandi et al. 2021). The development of the retina and eye is a complex, highly regulated process involving cellular interactions, signalling pathways, and transcription factors that ensure the precise formation of this crucial sensory organ. A profound understanding of the underlaying processes is a key aspect of the generation of therapeutic approaches. Therefore, useful tools and model systems where disease progression can be analysed and pharmaceutical drugs can be tested are always in demand.

Early Eye Development

Neuronal specification involves an interplay of many signalling pathways including WNT, FGF, BMP and retinoic acid leading to general patterning of the forming neural plate (S. W. Wilson and Houart 2004). A time dependent cascade of signalling events triggers the complex specification of the CNS. This is done by localised expression of these factors and their antagonists creating a balance regulating tissue identity. Protection of the presumptive anterior tissue from caudalizing factors can be achieved by morphogenic cell migration ahead of the gradient and antagonist expression. This mechanism is conserved in vertebrates even though the exact tissues guiding the process vary due to differences in embryonic organisation (Beddington and Robertson 1998). In mice, the source of antagonizing factors was identified as the anterior visceral endoderm (Kimura et al. 2000). This extra-embryonic tissue is localised on the distal tip of the embryo prior to gastrulation. The cells migrate unilaterally until they reach the border between the epiblast and the extra-embryonic ectoderm, thereby defining the anterior-posterior axis (Figure 1) (Srinivas et al. 2004). The cells are expressing, amongst others transcription factors, Hex, Hesx1, Lim1 and Cerberus-like, which repress the expression of posterior genes early, and therefore, limit the formation of the primitive streak to the posterior side (Belo et al. 1997). More precisely, the expression of Cerberus-like and Lefty1 restricts the primitive streak formation by antagonizing Nodal (Perea-Gomez et al. 2002). It was show that other specialised tissues e.g. the hypoblast in chick embryo are functionally similar to the anterior visceral endoderm in mice. Just like the anterior visceral endoderm it prohibits formation of multiple primitive streaks (Foley and Stern 2001; Bertocchini and Stern 2002). Interference with migration or signalling of the anterior visceral endoderm prevents proper patterning or even causes the loss of anterior CNS structures (Thomas and Beddington 1996; Shawlot et al. 1999; Martinez-Barbera and Beddington 2001).

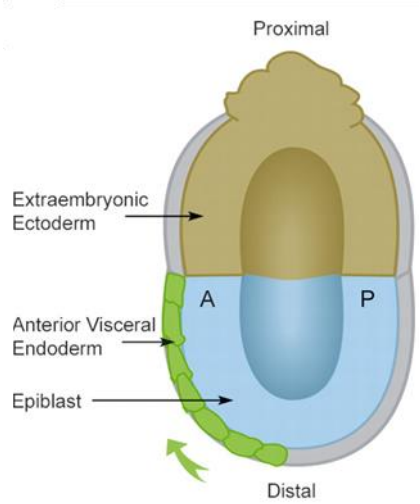


Figure 1: Movement of the anterior visceral endoderm cells induces anterior posterior patterning in the epiblast. *(Image from Srinivas et al. 2004)*

Members of the BMP family are proposed to be caudalizing factors and need to be antagonized for the correct formation and maintenance of the anterior neural plate (S. W. Wilson and Houart 2004). BMPs are expressed throughout the early epiblast. Increased BMP signalling can prohibit the formation of anterior neuronal tissue while the complete absence of BMP causes neural specification. Therefor BMP expression needs to be regulated at a low level where it allows neuronal induction. The neural fate was long seen as a default state (Hemmati-Brivanlou and Melton 1997). It was suggested that ectodermal cells will adapt neural fate if not exposed to BMP signalling (Muñoz-Sanjuán and Brivanlou 2002; S. W. Wilson and Houart 2004). Further research in amniotes showed that neither suppression of BMP nor are BMP antagonists sufficient or required to induce neuronal fate (Klingensmith et al. 1999; S. I. Wilson and Edlund 2001).

Prior to the secretion of the BMP antagonists Noggin or Chordin by the primitive node neural induction is already “pre” determined at the blastula stage (Stern 2002). FGF signalling is required for the initiation of neural induction, and thereafter, for suppression of BMP signalling (S. I. Wilson et al. 2000; Streit 2002; Akai and Storey 2003). This balance is further modified by Wnt signalling. In the chick embryo, Wnt regulates the fate decision between neural and epidermal identity. In the lateral epiblast Wnts are expressed and block the cells receptiveness to FGF signalling leading to Bmp expression and promotion of epidermal fate (S. Wilson et al. 2001). This early established pattern is then refined by local factor expression.

Later in development a gradient of Wnt is regulating the anterior posterior brain specification. Expression is high in the posterior part and needs to be antagonized in the anterior regions (Kiecker and Niehrs 2001; Nordström, Jessell, and Edlund 2002). Distinct levels of Wnt signalling regulate, for example, the formation of the telencephalon or the eyes. Shifting the gradient by inhibiting antagonists or adding ectopic Wnt signalling causes posterialization of the tissue truncating the prosencephalon and prohibit eye formation (van de Water et al. 2001; Houart et al. 2002; O. V. Lagutin et al. 2003).

Eye Field Specification

Development of the eyes begins with the establishment of the eye field within the anterior region of the neural plate, a flat layer of cells in the early embryo that will give rise to the nervous system. The eye field is a specialised area characterised by the overlapping expression of transcription factors that initiate the developmental program necessary to form the eyes (Zaghloul, Yan, and Moody 2005). Single transcription factors, essential for retinal development, are also expressed in adjacent tissues like Pax6 in the thalamus or Six3 in the hypothalamus. This exclusive expression pattern causes the regional restriction of cell fates (Byerly and Blackshaw 2009). The responsible eye field transcription factors (EFTF) are highly conserved in mammals and include Pax6, Rx, Six3, Six6, and Lhx2 (Zuber et al. 2003). They form a complex network and collectively contribute to the eye field's identity and its subsequent developmental processes. Therefore, they are associated with severe forms of ocular malformations including anophthalmia and microphthalmia. But since these factors also play critical roles in the broader context of CNS patterning and neurulation it was difficult to further investigate their distinct roles in eye development.

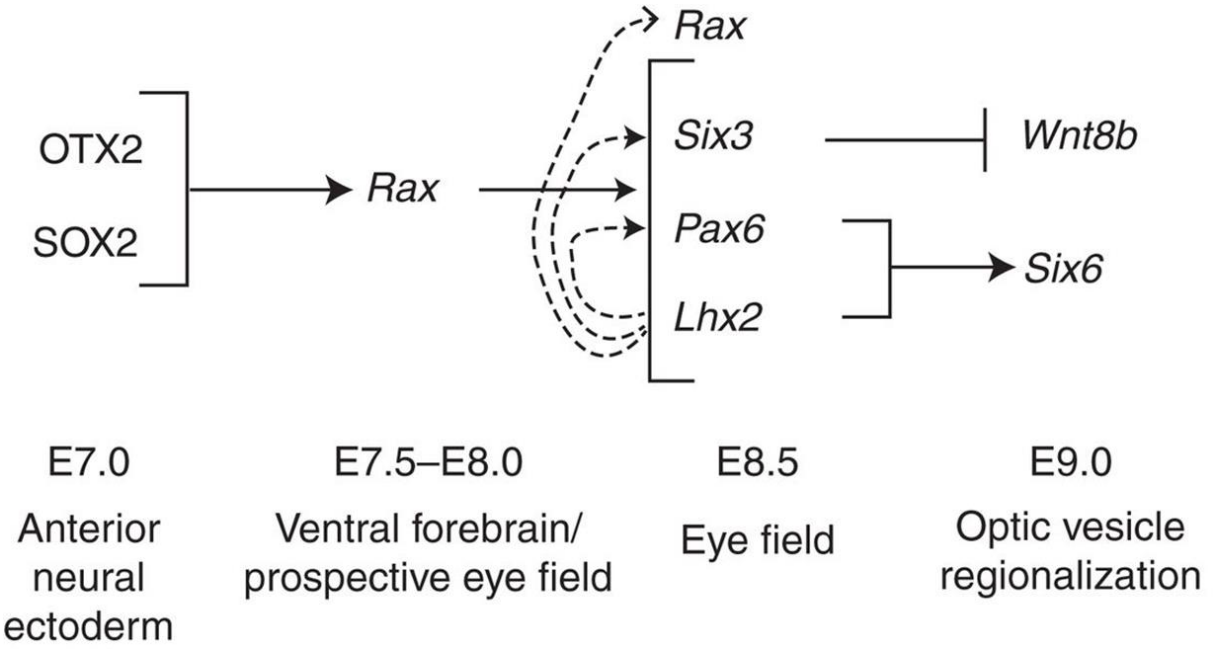


Figure 2: Transcription factors required for pre-specification and consolidating of the eye field identity at different developmental stages. *Expression of Otx2 and Sox2 pre-specifies the anterior neuroectoderm upon which Rx is expressed in the presumptive eye field. This leads to the eye field specification and the establishment of the transcriptional network consisting of the EFTFs Six3, Pax6, Lhx2 and Rx. Thereafter, local signalling determines the regional specification of the optic vesicle via factors like Six6 or Wnt signalling. (Image from Heavner and Pevny 2012)*

Otx2 is a bicoid-class homeobox domain gene expressed prior to eye field specification. It is expressed in the visceral endoderm and anterior neuroectoderm before gastrulation. Later in development, it gets restricted to the anterior side where it is involved in the induction of forebrain and midbrain. (Simeone et al. 1993; Ang et al. 1994; Rhinn et al. 1998). While heterozygous loss-of-function mutations cause ocular malfunctions, complete Otx2 null mice show severe head defects (Ragge, Brown, et al. 2005). By analysing additional causative genes for ocular malfunction, it was found that Otx2 together with the neural ectoderm transcription factor Sox2 induces Rx expression, which is

crucial for retinal development (Figure 2). Heterozygous loss-of-function mutations of Sox2 are known to cause bilateral anophthalmia supporting the theory that both factors play essential roles in the eye development (Fantes et al. 2003; Ragge, Lorenz, et al. 2005). Otx2 and Sox2 interact physically and binding of the conserved noncoding sequence upstream of Rx activates its transcription (Danno et al. 2008; Heavner and Pevny 2012). Thereafter, Otx2 is downregulated in the Rx expressing region of the early eye field (Andreazzoli et al. 1999).

The paired-like homeobox gene Rx is required to coordinate the expression of the EFTFs. $Rx^{-/-}$ mice are unable to upregulate the EFTFs for eye field specification. The expression of Otx2, Pax6 and Six3 in the anterior neural plate is not altered in $Rx^{-/-}$ mice but the upregulation in the presumptive eye field is lost. As a consequence these mice fail to develop optic vesicles (Mathers et al. 1997; L. Zhang, Mathers, and Jamrich 2000). Mutations in Rx can also be found in mutant mice strains with anophthalmia and some human patients (Tucker et al. 2001; Voronina et al. 2004). But Rx is not only essential for the upregulation of the EFTFs. In chimera experiments, where wild type and $Rx^{-/-}$ cells were mixed, only Rx^{+} cells contributed to retina formation (Medina-Martinez et al. 2009). This finding indicated a sorting mechanism that causes the Rx negative and Rx^{+} cells to segregate prior to morphogenesis of the ocular tissue. Therefore, it was suggested that cell-autonomous Rx expression is required for optic vesicle evagination (Rembold et al. 2006; Medina-Martinez et al. 2009). After fulfilling its task to coordinate the eye field formation and upregulation EFTFs, Rx remains expressed and plays a considerable role in the maintenance and proliferation of RPCs. Over-expression of Rx in *Xenopus* and zebrafish lead to over-proliferation of retinal cells and can ectopically induce expression of markers for undifferentiated progenitors (Casarosa et al. 2003; Bailey et al. 2004).

The homeobox gene Pax6 was long time postulated as the master regulator of eye development. This is because it is a highly conserved gene and involved in varying processes over the course or retinal development (van Heyningen and Williamson 2002). In was even shown that misexpression of Pax6 in *Xenopus* lead to the formation of ectopic eye structures (Chow et al. 1999). Orthologs of Pax6 have been identified in many species (Krauss et al. 1991; Walther and Gruss 1991; Püschel, Gruss, and Westerfield 1992; Hirsch and Harris 1997). Mice, that lack Pax6 function, show normal upregulation of Rx in the eye field and formation of RPCs Pax6 expression in turn is dependent on functional Rx expression (L. Zhang, Mathers, and Jamrich 2000). But besides its function in the early development Pax6 expression is maintained in the retina, lens and cornea (W. Zhang et al. 2001). Therefore, mutations in the Pax6 gene and misexpression of Pax6 causes several ocular malfunctions.

Small eye (*sey*) mice have a semi dominant mutation in the Pax6 gene (Hill et al. 1991). Heterozygote mice suffer from iris hypoplasia while homozygote mice lack eyes and nasal cavities and exhibit brain abnormalities often dying soon after birth (Hogan et al. 1988). It was shown that *sey* mice mimic the most common mutation responsible for aniridia in humans which is also associated with mutations of the Pax6 gene (Hickmott et al. 2018). Therefore, these mice are used as disease models for human aniridia (van der Meer-de Jong et al. 1990; Glaser, Lane, and Housman 1990). On the other hand overexpression of Pax6 also leads to severe eye abnormalities indicating its function is dose dependent and could even require a threshold concentration for proper eye development (Hill et al. 1991; Schedl et al. 1996).

Six3 is a homeobox containing transcription factor closely related to *sine oculis* in *Drosophila* (Oliver et al. 1995). Like other EFTFs it is expressed in the anterior neural plate before specializing its expression to the olfactory placode, optic vesicles, lens placode, midbrain and ventral forebrain (L. Zhang, Mathers, and Jamrich 2000). In the optic vesicles, Six3 and Rx regulate progenitor proliferation (Bailey et al. 2004). More importantly, Six3 regulates two major signalling pathways at distinct timepoints of the early eye development making it essential for proper eye development.

Firstly, it functions as a direct negative regulator for Wnt, and is therefore involved in the establishment of the gradient subdividing the rostral neural plate. Increased Wnt is sufficient to supress Six3 expression leading to a negative feedback loop (Braun et al. 2003). As mentioned earlier, the specification of the eye field requires Wnt signalling at a well-defined dosage. It needs to be an intermediate between the concentrations in the prospective telencephalic and diencephalic region (Mukhopadhyay et al. 2001; Houart et al. 2002). In Six3 null mice, Wnt expression was expanded into the anterior region causing a reduction of the prosencephalon (O. V. Lagutin et al. 2003). The canonical deletion of Six3 caused a Wnt8b-mediated arrest of neural retina specification in mice (Figure 2) (W. Liu et al. 2010). These studies demonstrate the importance of the Six3-mediated antagonizing of Wnt to stabilize its signalling gradient. In accordance to this, ectopic expression of Six3 in mid- or hindbrain regions, where Wnt levels are normally too high for retinal induction, causes the generation of ectopic retinal tissue in fish and $Pax6^{+}$ ectopic vesicles mouse embryos (Loosli, Winkler, and Wittbrodt 1999; O. Lagutin et al. 2001).

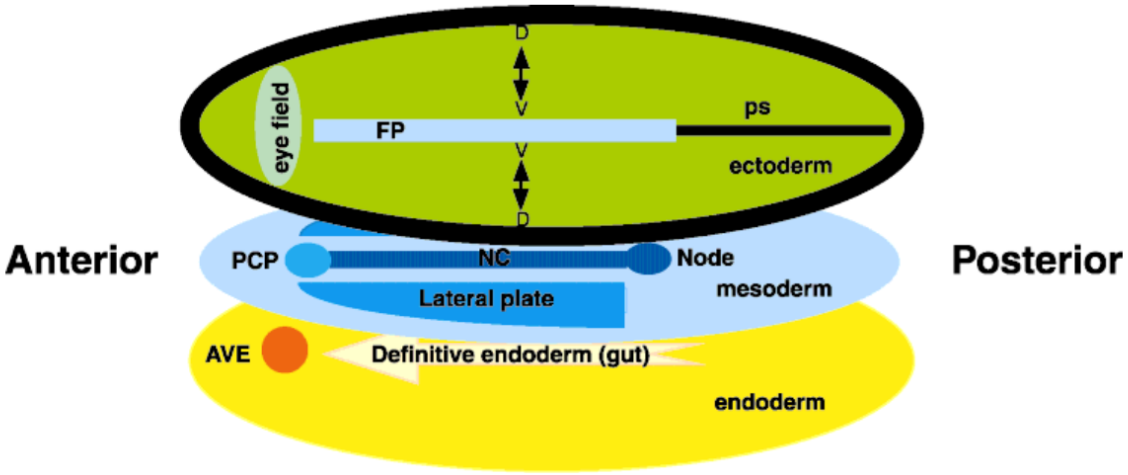


Figure 3: Schematic depiction of the tissue organisation underlaying the eye field and their signalling centres. *AVE: anterior visceral endoderm; D-V: dorsal to ventral; FP: floorplate; NC: notochord; PCP: prechordal plate; ps: primitive streak. (Image from Roessler and Muenke 2001)*

Besides its function in eye field specification Six3 is thereafter required for proper eye field segregation. The partitioning of the eye field to form two distinct lateral eyes is essential and dysregulation of this process can cause cyclopia. This process is mediated by axial tissue like the presumptive hypothalamus and underlying mesoderm (Figure 3). In zebrafish, the migration of diencephalic precursor cells separates the eye field into the two lateral regions. These prospective hypothalamic cells move anteriorly displacing the cells of the eye field while in mutants, where the retina remains fused these cells failed to migrate (Varga, Wegner, and Westerfield 1999). Additionally, the removal of the prechordal mesoderm leads to the formation of a single retina field indicating that suppression of the retinal fate in the median region causes the specification of two distinct lateral eye fields (Li et al. 1997). This is mediated by suppression of Pax6 by sonic hedgehog (Shh) signalling from the prechordal plate (Chiang et al. 1996; Hayhurst and McConnell 2003). Shh is a secreted morphogen that is expressed along the axial midline thereby inducing the floorplate in the neural tube (Echelard et al. 1993). And after its induction the floorplate and the anterior forebrain act as additional signalling centres for Shh expression. (Roessler and Muenke 2001) In accordance to this loss-of-function of Shh

causes cyclopia or other midline abnormalities in vertebrates (Roessler and Muenke 2000; Muenke and Beachy, n.d.; Nasevicius and Ekker 2000).

In mice, Six3 directly activates Shh in the rostral diencephalon ventral midline. Conversely, Shh maintains Six3 expression leading to a positive feedback dynamic between these factors (Jeong et al. 2008; Geng et al. 2008). Mutations in Six3 cause ocular defects like cyclopia and are a known cause for holoprosencephaly in humans (Cohen Jr. 2006). The Six3 mutation associated with holoprosencephaly led to decreased activity of Six3 in a fish model. Lowering the transcription factors activity had the same effect as reduced amounts of intact Six3 which lead to a reduction of Shh (Geng et al. 2008). This means, when the amount of functional Six3 is altered, it fails to activate Shh expression which in turn causes to holoprosencephaly (Chiang et al. 1996).

Taken together these findings show the complexity of retinal development *in vivo* and that a plethora of strictly time and dose dependent factors and signalling pathways are involved in the specification of the eye field. Several stages of pre-specification are required before the EFTFs establish the transcriptional network that consolidates the identity of the eye field.

Morphogenesis of the Optic Cup

The first visible morphological feature of eye development are the bilateral optic sulci (Heavner and Pevny 2012). As the neural plate folds to form the neural tube, the eye field cells are induced to form bilateral optic vesicles, which bulge outward from the diencephalon around E9 (Figure 5). This evagination is critical and serves as the foundation for all major ocular structures (Adler and Canto-Soler 2007). Once formed, the optic vesicles undergo a complex process of regional specification. It can be partitioned into the distal part that will become the neural retina, the dorsal region with the presumptive RPE and the ventral region where the optic stalk is formed (Yun et al. 2009). These regions are defined by the expression of additional transcription factors and signalling molecules, suggesting that the RGCs of the early optic vesicle are not yet determined but hold the potential to differentiate according to the signals their surrounding provides (Heavner and Pevny 2012).

The presumptive neural retina in the distal region expresses Vsx2 and Pax6 and gets determined upon contact with the surface ectoderm (Figure 4 A). Here FGF plays an important role in the initialization and differentiation of the neural retina. FGF-8 is expressed in the distal optic vesicle upon contact with the ectoderm and remains expressed in the neural retina upregulating Vsx2 expression (Vogel-Höpker et al. 2000). In absence of the surface ectoderm, which is the main source of FGF, Vsx2 expression is decreased and the epithelium is converted into RPE (Nguyen and Arnheiter 2000). Additionally, in mice lacking FGF9 the RPE extends into the presumptive neural retina (S. Zhao et al. 2001). On the other hand, ectopic addition of bFGF in the eye causes the presumptive RPE to transdifferentiate into neural retina *in vivo* and *in vitro* (C. M. Park and Hollenberg 1989; Pittack, Jones, and Reh 1991; S. Zhao, Thornquist, and Barnstable 1995). Therefore, FGF signalling is regulating the border between the two tissues and does this via downregulation of the basic helix loop helix leucine zipper protein MITF through its repression by Vsx2 (Horsford et al. 2005).

Initially, WNT, BMP and TGFβ signalling specifies the presumptive RPE which is characterised by Mitf and Otx2 expression (Figure 4 B) (Westenskow, Piccolo, and Fuhrmann 2009; Steinfeld et al. 2013; Ma et al. 2019). Extra-ocular mesenchyme inhibits the expression of the neural retina specifying factor Vsx2 and downregulates the expression of Pax6 and Six6 in the dorsal optic vesicle (Fuhrmann, Levine, and Reh 2000). This leads to the upregulation of Mitf in this region, which in turn downregulates Pax6

and therefore helps to maintain RPE fate (Mochii et al. 1998). This coincides with the complementary expression patterns of Pax6 and Mitf. The mesenchymal signalling is mediated by the secretion of the TGFβ family member Activin. While in absence of the extraocular mesenchyme RPE did not development in embryonic chick, Activin is able to substitute for the mesenchyme promoting RPE specific gene expression (Fuhrmann, Levine, and Reh 2000). Absence of Mitf causes loss of pigmentation, hyperproliferation and subsequently development of a second laminated retina that is structurally inverted. Both retinae degenerate and the eyes are not functional (Hodgkinson et al. 1993; Bumsted and Barnstable 2000).

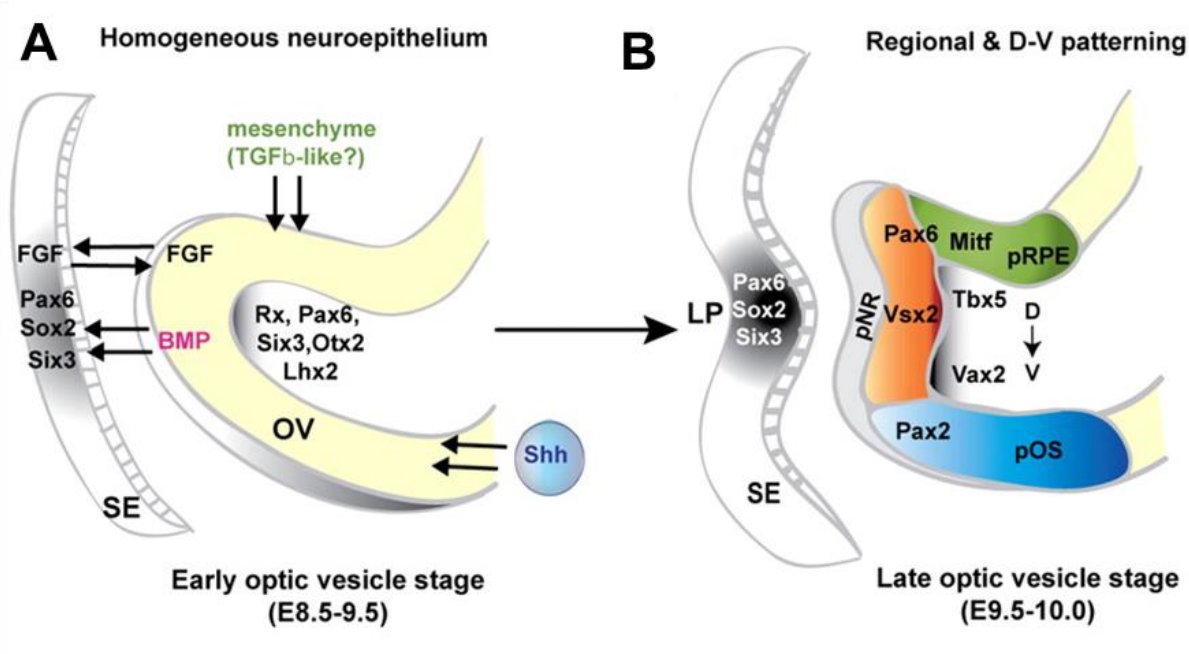


Figure 4: Compartmentalization of the optic vesicle by interaction of intrinsic and extrinsic signalling cues. *The optic vesicle expresses the transcription network of the EFTFs and is compartmentalized upon signalling cues from adjacent tissue. These include FGF from the surface ectoderm, Shh on the ventral side and TGF-β from the extra ocular mesenchyme (A). Upon these signals, specialised region form: The Mitf expressing presumptive RPE on the dorsal side, the Pax6 and Vsx2 expressing presumptive retina on the anterior side towards the lens placode and the Pax2 expressing presumptive optic stalk on the ventral side (B). D-V: dorsal to ventral; LP: lens placode; pOS, presumptive optic stalk; pNR, presumptive neural retina; pRPE, presumptive retinal pigment epithelium; SE: surface ectoderm. (Image adapted from Yun et al. 2009)*

Vax family members establish the general dorso-ventral patterning in the late optic vesicle (Mui et al. 2002). The anti-retinogenic homeodomain gene Vax2 is expressed mainly ventral with a gradient towards the dorsal side, where Tbx5 is expressed (Barbieri et al. 1999; Koshiba-Takeuchi et al. 2000). The two factors supress each other and their interplay enables the graded expression patterns of Ephs and Ephrins that are essential for retinotectal projection (Koshiba-Takeuchi et al. 2000; Yang 2004).

The Vax2 paralog Vax1 is expressed ventrally and participates in the establishment of the border between the presumptive neural retina and the presumptive optic stalk by negative regulation of Pax6 and Rx expression that characterise the retinal fate (Hallonet et al. 1999; Take-uchi, Clarke, and Wilson 2003). Shh signalling definiens the future optic stalk mainly by induction of Pax2 expression in the ventral region and simultaneous inhibition of Pax6 expression (Nornes et al. 1990; Macdonald et al. 1995; Yang 2004). But Vax1 seems to act in parallel with Pax2 as downstream elements of Shh (Take-uchi, Clarke, and Wilson 2003). Therefore increased Shh signalling causes an increase of Pax2 and Vax1 expression in fish and chick embryos, while the Pax6 expressing region is reduced causing phenotypes

with small eyes and an enlarged optic stalk (Ekker et al. 1995; X.-M. Zhang and Yang 2001b). Pax6 and Pax2 show reciprocal transcriptional repression of each other, further strengthening the border between retina and optic stalk (Schwarz et al. 2000).

The optic vesicles, driven by signals from both within and outside the eye field, begin to invaginate to form the optic cup, a double-layered structure that differentiates into the retina and the retinal pigment epithelium. The inner layer of the optic cup will become the neural retina while the outer layer forms the RPE (Heavner and Pevny 2012). Experiments *in vitro* showed spontaneous optic cup formation in ROs (Eiraku et al. 2011). These were free-floating cell aggregates without contact to surface ectoderm suggesting the invagination can be initiated intrinsically without external signals from surrounding tissue (Eiraku et al. 2011).

One important signalling molecule for the invagination of the optic cup is retinoic acid (RA) (Cvekl and Wang 2009). Retinaldehyde dehydrogenases produce RA from Vitamin A and are involved in dorsal and ventral invagination of the optic vesicle (Mic et al. 2004). There are three retinaldehyde dehydrogenases involved in the eye morphogenesis, Raldh1, Raldh2 and Raldh3 which have distinct expression patterns (Grün et al. 2000). Raldh2 is the main producer of RA responsible for the invagination of the optic vesicle (Mic et al. 2004). First, it is expressed in the optic vesicle, but by the time of invagination Raldh2 expression is restricted to the temporal mesenchyme. In addition, Raldh3 expression in the RPE is responsible for the invagination of the ventral retina (Molotkov, Molotkova, and Duester 2006). Because of its late expression and low enzyme activity Raldh1 is most likely responsible for the regulation of periocular mesenchymal growth and other possible roles in the adult dorsal retina (Fan et al. 2003).

Due to the invagination of the optic vesicle the optic stalk and the ventral retina get into contact and form the optic fissure that is later required for the exiting optic nerve and the entrance of blood vessels into the retina (Saint-Geniez and D'Amore 2004).

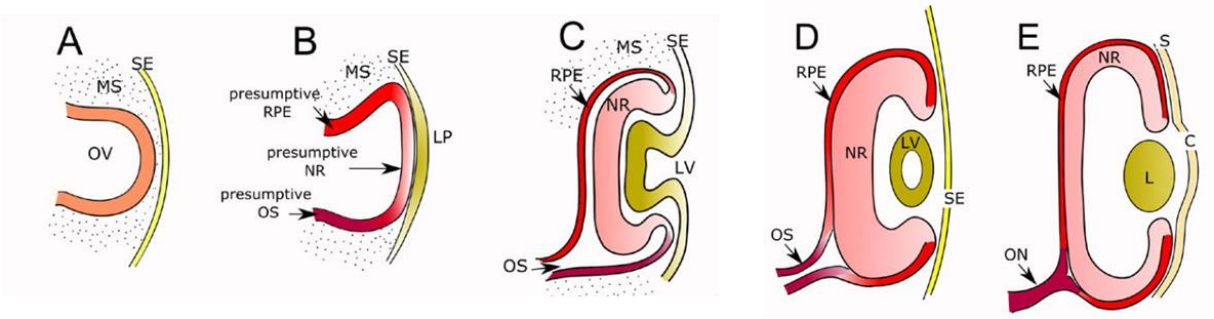


Figure 5: Morphogenic development of the optic vesicle into the optic cup*. The optic vesicle evaginates until it gets into contact with the surface ectoderm (A). Thereafter, the lens placode forms and simultaneously the compartmentalization of the optic vesicle begins (B). Invagination of the vesicle leads to the formation of a bilayered cup with the presumptive retina on the inside and presumptive RPE on the outside. The optic stalk is formed ventrally and later on contains the optic nerve. Simultaneously, the lens placode evaginates to for the lens vesicle from which the lens is differentiated (C-E). C: cornea; L: lens; LP: lens placode; LV: lens vesicle; MS: mesenchyme; NR: neural retina; ON: optic nerve; OS: optic stalk; OV: optic vesicle; RPE: retinal pigment epithelium; S: sclera; SE: surface ectoderm. (Image adapted from Adler and Canto-Soler 2007)*

Simultaneously, the lens placode forms in the surface ectoderm expressing Sox2, Pax6 and Six3 (W. Liu et al. 2006). This process is mediated by BMP and FGF signalling which both require ligand secretion from the optic vesicle (Faber et al. 2001; Furuta and Hogan 1998). For example BMP signalling initiated by Lhx2 expression in the optic vesicle regulates Sox2 expression in the presumptive lens tissue (Furuta

and Hogan 1998). The interaction of Pax6 with Sox2 and Six3 regulates the development and maintenance of different ocular tissues, including the corneal epithelium and the lens (Smith et al. 2009). Therefore, the heterozygous mutation of Pax6 resulted in failed lens formation and stopped retinal development at the stage of the optic vesicle (Ashery-Padan et al. 2000). Sox gene expression works in parallel and is additionally needed in the Pax6 expressing ectoderm. Sox1, Sox2 and Sox3 are all expressed in the lens tissue and while Sox2/3 are responsible for the expression of crystallins, Sox1 is mainly needed for the further development of the lens (Kamachi et al. 1998). Later the lens placode begins to invaginate to form the lens vesicle (Figure 4 A). This structure will detach from the ectoderm and differentiate into the lens which is necessary to focus light on the retina (Figure 5 D and E) (Adler and Canto-Soler 2007).

The differentiation of cells within the optic cup is tightly regulated by a network of transcription factors and influenced by extrinsic signals from neighbouring tissues and intrinsic factors within the cells. These signalling interactions ensure that the cells within the developing eye acquire the correct identity and function, critical for the precise assembly of the ocular tissues.

The Neural Retina

The neural retina is a laminated structure that comprises the six major neuronal cell types rods, cones, amacrine-, horizontal-, bipolar- and ganglion cells (Dowling 1987). Each of which has specialised tasks and resides in specific retinal layers only allowing contact to certain other neurons to establish the complex neuronal circuit needed for proper vision. Together with one glia cell type, the Müller glia cell (MGC), these cells derive from a common pool of multipotent RPCs (Turner and Cepko 1987; Wetts and Fraser 1988). Including all subtypes, the retina consists of over 50 distinct cell types (Masland 2012). They form a complex network that enables us to detect signals from our surrounding process the information and transmit it further to the brain (Figure 6).

To structure such a complex network, the retinal cells are segregated into two major types of layers. The nuclear layers where the cells nuclei are located and the plexiform layers where synapses are formed (Dowling 1987). The most apical nuclear layer is the outer nuclear layer. Here, rod and cone photoreceptors are located. In apical direction the neural retina is limited by the outer limiting membrane and only the outer segments of the photoreceptors forming the photoreceptor layer reach out in the direction of the RPE with which they are in direct contact. Photoreceptors can be divided in two major classes. The cones, that are more sensitive and responsible for low light vision and express rhodopsin. And the rods, that mediate chromatic vision and express opsins that are tuned to different wavelengths. Humans have three opsins, The S-opsin, M-opsin and L-opsin that refer to responsiveness to short, medium or long wavelengths of incoming light (Yan et al. 2020). The photoreceptors form synapses in the outer plexiform layer. Here they form connections with interneurons like bipolar cells and horizontal cells (Gollisch and Meister 2010).

Bipolar cells reach into the inner and outer plexiform layer connecting photoreceptors and RGCs. In mice, bipolar cells constitute about 41% of the cells in the inner nuclear layer, and are therefore, the most common interneuron in the mammalian retina (Jeon, Strettoi, and Masland 1998). They are categorized in three classes depending on the photoreceptor which they form synapses with and the stimulus responsible for action potential firing (Masland 2012). Bipolar cells that are excited when a light stimulus increases are called ON-bipolar cells and can be triggered by rods and cones, while OFF bipolar cells get excited by lowering light conditions and are connected to cones (Greferath, Grünert, and Wässle 1990; B. B. Boycott and Wässle 1991). Photoreceptors release glutamate under dark

conditions that causes the depolarization of OFF bipolar cells and hyperpolarization of ON bipolar cells (Boccuni and Fairless 2022). Increased light stimulus causes the opsin activation that results in G-protein-mediated activation of a phosphodiesterase that cleaves cGMP (Shiells and Falk 1997). Therefore, more cGMP-mediated Na channels get blocked due to lower levels of cGMP. This causes the hyperpolarization in the photoreceptor, and therefor, decrease of glutamate release.

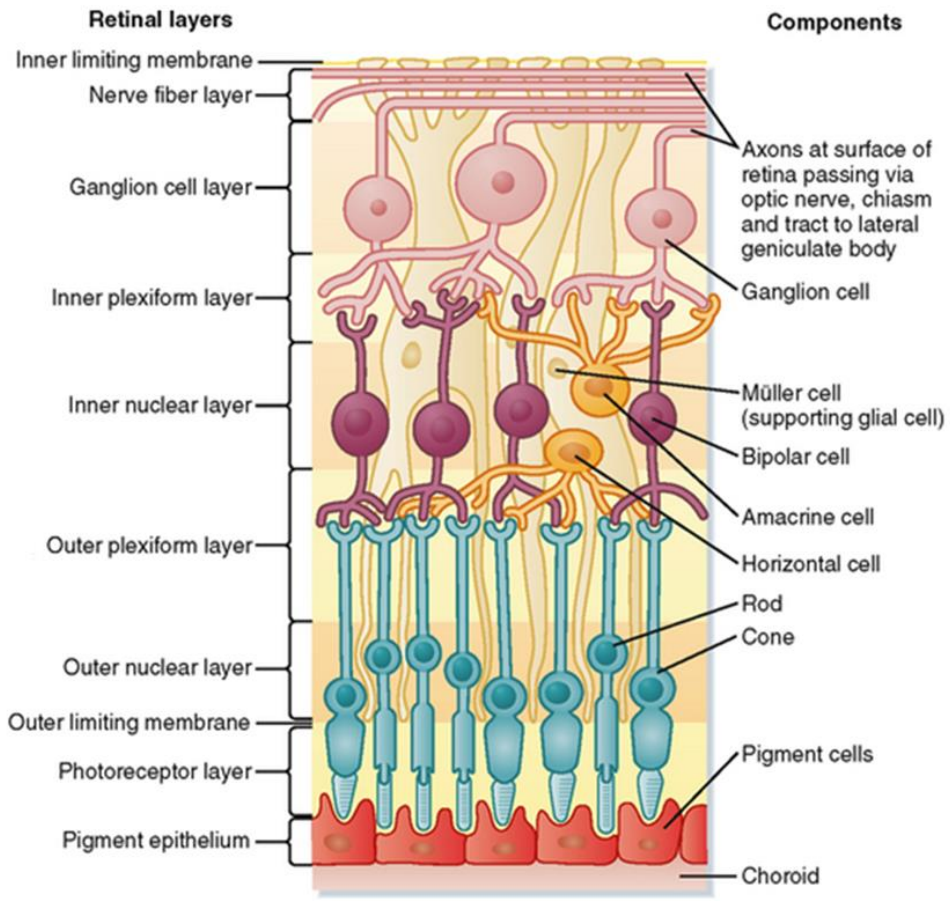


***Figure 6: Cross-section throughout the retina**. Schematic overview over the retinal organisation and its subdivision into multiple layers and the positioning of the seven basic cell types in the tissue. (Image from Koeppen and Stanton 2023)*

Not all signals are directly transmitted onto the ganglion cells. Horizontal cells and amacrine cells are inhibitory neurons with synaptic connection restricted to one plexiform layer for processing and finetuning of the neuronal circuits. There are two distinct types of horizontal cells in mammals, except for mice and rats which have only one (Kolb, Mariani, and Gallego 1980; Brian Blundell Boycott, Hopkins, and Sperling 1997; Masland 2012). They account for less than 5% of the cells in the inner nuclear layer and directly give feedback to the photoreceptors, thereby increasing the contrast between bright and darker regions. Horizontal cells are connected to multiple photoreceptors, and therefore, measure illumination over a broader region (Chapot, Euler, and Schubert 2017; Chaya et al. 2017). The illumination across this area contains few information which is why the horizontal cells depending on the signals that emerges inside their receptive field transmit or inhibit signals (Yan et al. 2020). Hereby, they contribute to the clearer processing of contrast by the RGCs that differentiate between signals that are classified as in the “centre” region directly above the RGCs or the “surround” structure where peripheral connections are formed.

Amacrine cells can be divided into 29 subclasses and are the main inhibitory neuron (Masland 2012). In contrast to horizontal cells, that make up 3% of the INL, amacrine cells outnumber them by far, reaching 39% in mice (Jeon, Strettoi, and Masland 1998). Most signals transmitted to the RGCs are not directly passed on by bipolar cells but amacrine cells (Calkins et al. 1994; Jacoby et al. 1996; Bloomfield and Dacheux 2001). They mainly act via GABA and glycine neurotransmitters but are not restricted to those as some amacrine cells express none of these (Yan et al. 2020). Since this group shows so much variety, multiple specialised tasks are targeted by amacrine cells resulting in the control and modulation of ganglion cell responses.

Lastly, RGCs form the ganglion cell layer where only RGCs and some displaced amacrine cells reside in (Haverkamp and Wässle 2000; Amini, Rocha-Martins, and Norden 2018). They receive information in the inner plexiform layer and transmit the signals via the optic nerve to the brain for further processing (Koeppen and Stanton 2023). The mammalian retina has 15 types of ganglion cells involved in distinct visual perception. These include movement, colour or contrast information that is each processed by a specialised circuit in the retina (Wässle 2004).

Taking a closer look, the synaptic layers can be subdivided in additional strata used for interconnections between specific subtypes of neurons (Haverkamp and Wässle 2000). Projecting in the same region facilitates the correct wiring of the neuronal circuits. During development, not only spatial but also timely separation supports this sorting mechanism. It is known that the differentiation of the retinal neurons follows a distinct time dependent differentiation pattern conserved in vertebrates (Figure 7) (C. Cepko 2014). Injections of the radioactive nucleoside ^{3}H-thymidine at different timepoints showed the generation of a subset of postmitotic neurons dependent on the time of injection (R. W. Young 1985; Turner, Snyder, and Cepko 1990). It was identified that the murine retinal cell types were generated in two major waves of histogenesis. The first wave generates the early born neurons like RGCs, horizonal cells cones and the majority of amacrine cells. In the second wave, that extends into the postnatal stage, the later born neurons bipolar cells and rods were born. Additionally, MGCs, which are the last cell type to arise were generated (R. W. Young 1985).

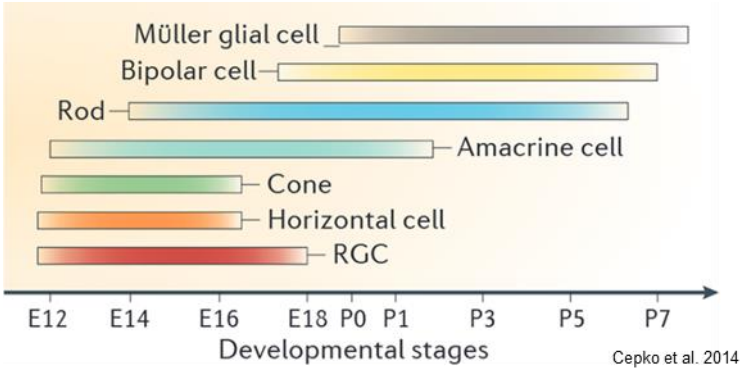


Figure 7: Conserved birth order of retinal cell types derived from a common RPC pool. *The generation of retinal neurons can be divided in early born neurons like RGCs, horizontal cells , cones and amacrine cells, which have their differentiation peak prior to birth and the late born neurons including rods, bipolar cells and MGCs that develop mainly postnatally. (Image adapted from C. Cepko 2014)*

Early lineage tracing experiments showed that the two daughter cells of one RPC further divide and a progenitor is not limited to one lineage. One RPC can contribute to almost all cell type populations in the retina, and is therefore, not restricted to a fate by lineage or time alone (Holt et al. 1988; Turner, Snyder, and Cepko 1990). It was proposed that the RPCs go through irreversible stages of competence

that change their ability to generate a certain type of neuron during this time (C. L. Cepko et al. 1996; C. Cepko 2014). This mechanism is regulated by intrinsic and extrinsic factors since secreted factors can influence cell fate determination *in vitro* (Belliveau and Cepko 1999). It was shown that at least for the later born cell types intrinsic limitations regulate cell fate even in an altered environment (Belliveau and Cepko 1999; Cayouette, Barres, and Raff 2003). The final cell fate decision of a neuron is therefore influenced by the intrinsic competence of the RPC on a gene expression level and extrinsic factors contributing to the differentiation process (Livesey and Cepko 2001). These findings suggest that RPCs can give rise to a limited variety of neurons at each timepoint but these can be directed by external signals including Shh, RA or members of the ciliary neurotrophic factor (CNTF) or leukaemia inhibitory factor (LIF) family (Holt et al. 1988; Livesey and Cepko 2001). However, there is no strictly synchronized time dependent change of competence, since there is great overlap in the periods of generation for several neurons (la Vail, Rapaport, and Rakic 1991; Stiemke and Hollyfield 1995).

One example, which is extensively studied, is the generation of RGCs. First, the retina consists only of proliferating progenitor cells and competent progenitors to generate RGCs represent the majority of the embryonic retina. Competence within this group is determined by Notch signalling while differentiation is additionally regulated by FGF and Shh signalling (Austin et al. 1995). Studies *in vivo* and *in vitro* showed that Notch signalling maintains the RPC fate and is therefore inversely related to ganglion cell specification (Austin et al. 1995). The differentiation of this progenitor pool is initiated in the central retina when a wave of Shh signalling induces the RPCs to give rise to postmitotic neurons (Yang 2004). In chick embryos inhibition of FGF signalling during this developmental phase inhibited RGC formation (McCabe, Gunther, and Reh 1999). In contrast to findings in drosophila in the vertebrate retina no cell cycle synchronisation takes place at the wave front for differentiation. Nevertheless, some mechanisms seem to be conserved among species. Shh signalling is maintained in the differentiated RGCs causing the differentiation to progress (Heberlein and Moses 1995; Neumann and Nuesslein-Volhard 2000). Differentiated RGCs are not required for further differentiation of RPCs and behind the wave front they even negatively regulate differentiation by the accumulation of Shh. This mechanism was confirmed by overexpression studies were RGCs numbers were reduced *in vivo* and *in vitro*. Conversely, the antibody-mediated reduction of availability of Shh caused increased RGCs numbers (X.-M. Zhang and Yang 2001a). Additionally, older retinal cells secrete factors that inhibit RGC differentiation limiting their differentiation to the earliest timepoint (Waid and McLoon 1998).

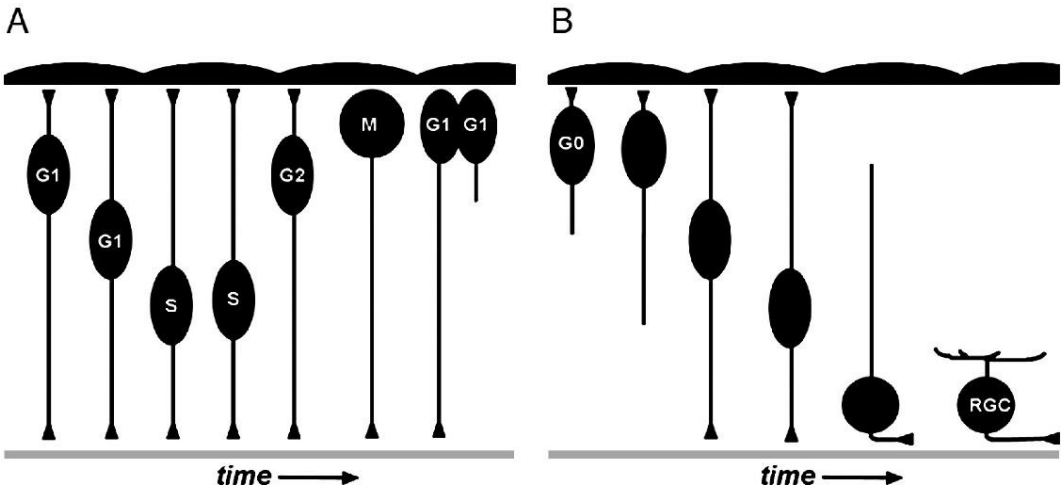


Figure 8: Schematic depiction of interkinetic nuclear migration and nuclear translocation. *Cell cycle dependant interkinetic nuclear migration of a RPCs throughout the epithelium (A). Post-mitotic nuclear translocation is exemplarily depicted for a RGC translocating from the apical to the basal side (B). (Image from Baye and Link 2008)*

Time dependent differentiation of neuronal cell types alone does not form the complex retinal structures that enables proper vision. More important is each neurons localisation in the epithelium to form the right connections and fulfil its task in the circuit.

In the retina, just as in other areas of the CNS, nuclear migration plays an important role for proliferation and cellular organisation. Many examples show the presence of interkinetic nuclear migration in the majority of neuroepithelia and even other polarized cells (F. C. Sauer 1936; Bhide 1996; Takahashi et al. 1999; Nowakowski et al. 2002). These nuclear movements are synchronized with the cell cycle. While the S-Phase takes place more basally, the M-Phase is on the apical side (Figure 8 A). Mitosis is always limited to the apical region of the retina that is in direct contact with the RPE resembling the equivalent of the ventricular zone in the CNS (Robinson, Rapaport, and Stone 1985). During interkinetic nuclear migration the cells maintain apical and basal processes spanning the retina. This resembles a pseudostratified tissue where only the nuclei translocate during the cell cycle. These movements were confirmed by two important observations. One is, that mitotic inhibitors caused arrest of metaphase cells only at the apical side. Meanwhile pulse-labelling with ^{3}H-thymidine resulted in stained cells all over the epithelium (M. E. Sauer and Walker 1959; M. E. Sauer and Chittenden 1959; Langman, Guerrant, and Freeman 1966). After the division, one cell keeps the basal process while the daughter cell forms a new basal process (Baye and Link 2008).

Interkinetic nuclear migration is needed for the regulation of RPCs proliferation. It is thought to be a control mechanism to keep a proliferative population of cells from contact with differentiating neurons and postmitotic neurons as demonstrated in a mathematical model (Murciano et al. 2002). Cell-cell contact-mediated signalling via local factors and Notch are relevant for the fate decision if cells stay proliferative or differentiate (Henrique et al. 1995; Lewis 1998). Neural precursor cells are known to produce delta to inhibit Notch in neighbouring cells and supress neuronal fate via lateral inhibition (Heitzler and Simpson 1991; Ghysen et al. 1993; Henrique et al. 1995; Lewis 1998). It was also discussed that gap junctions modulate the interkinetic nuclear migration to influence proliferation. Undifferentiated cells form a network that excludes differentiated neurons where gap junction-mediated signalling could alter the translocation speed (Pearson et al. 2004; Weissman et al. 2004; Pearson et al. 2005). Migration time, therefore, regulates the duration of the cell cycle and thereby proliferation (Pearson et al. 2005).

The birth of a certain cell type examined by ^{3}H-thymidine labelling is defined when they undergo their last S-phase (C. L. Cepko et al. 1996). Thereafter, they exit the cell cycle and move to their final location inside the epithelium by a different kind of locomotion, nuclear translocation (Figure 8 B) (Baye and Link 2008). This process is characterised by rapid extension of a long basal process then followed by rapid retraction. Migration happens in the direction of the leading neurite and simultaneously the trailing process is retracted leading to a lacrimal shape of the migrating cell (Tsai and Gleeson 2005). The cytoskeleton is heavily altered during this process. Perinuclear microtubules form a cage that surrounds the nucleus and holds it at the trailing zone of the cell. (Rivas and Hatten 1995). Simultaneously, microtubules project into the leading process. In the CNS, both types of microtubules emerge from the centrosome that is positioned between the leading process and the nucleus. It is always aligned with the nucleus on the side with the leading process, and can therefore, serve as an indicator for the direction of the movement (Tanaka et al. 2004). First, the centrosome moves forward and the nucleus follows generating a “two-stroke” movement (Solecki et al. 2004). In the retina, however, as seen in RGCs, nuclear translocation is seemingly not centrosome driven but by the basal process, since it remains lateral or apical during basal locomotion (Hinds and Hinds 1974; Zolessi et al. 2006). The mechanism is guided by stabilized microtubules and basal process attachment (Icha et al. 2016).

In the CNS, the neurons often use radial glia as guiding structures for their migration but they do not depend on them (Ghashghaei, Lai, and Anton 2007; Nadarajah 2003). Actin is accumulated in the trailing zone and forms a cortical ring. Additionally, it is enriched in short filopodia that are forming all over the leading zone and create junctions beneath the soma (Hatten 2002). Treatment with cytochalasin B inhibited the migration leading to the conclusion that actin subunit assembly is required for the movement (Rivas and Hatten 1995). Actin could be part of the complex involved in the anchoring and force generation of the dynein motor proteins associated with the microtubules (Schroer 2004). Dynein proteins are localised on the base of the microtubule plus end on the nucleus and the substrate junctions in the leading process (Tsai and Gleeson 2005). These include in both cases Lis1, NudC and dynactin which contains actin as well as actin related protein (Arp1) (Aumais et al. 2001; Tanaka et al. 2004). Arp proteins and actin were folded by cytoplasmic chaperonin and were shown to interact *in vivo* (Melki et al. 1993; Eckley and Schroer 2003). The cytochalasin could possibly disrupt this complexation of proteins including actin, and therefore, the migration is stopped.

In the early development RPCs undergo symmetric division to produce more PRCs rather than postmitotic neurons. During symmetric divisions only one of the daughter cells inherits the basal process, while the other cell extends a new process. Interestingly, this behaviour results in asymmetric behaviours in cell cycle length, interkinetic nuclear migration and progeny (Saito et al. 2003). Later, RPCs shift to asymmetric divisions where one cell remains as RPC continuing to divide and the other daughter cell exits the cell cycle and differentiates like it is known from cortical cells (Mione et al. 1997; W. Zhong et al. 1996). During late development symmetric divisions result in the production of only postmitotic neurons (Livesey and Cepko 2001).

As an adjustment of the competence model of retinal cell differentiation, it was found that some neurons were generated by committed progenitors (C. Cepko 2014). This results in heterogeneity of the RPC population at several time points. For example, amacrine and horizontal cells, which have very close maxima of their timepoints of birth, are generated from a transcriptionally distinct progenitor subgroup expressing VC1.1 and syntaxin at that state (Alexiades and Cepko 1997). It was shown that $VC1.1^{+}$ progenitors give mainly rise to amacrine and horizontal cells during early development. While in parallel $VC1.1^{-}$ progenitors generated cones during this period (Alexiades and Cepko 1997). Other examples are the basic helix-loop-helix (bHLH) genes that regulate progeny of RPCs. These include Math5 in early retinal development or Mash1 that is expressed in a subpopulation associated with late retinal cell fates like bipolar cells and MGCs (Jasoni and Reh 1996; Brown et al. 1998). A balance of proneural and antineuronal bHLH genes mediated by Notch regulates the gliogenic switch that generates MGCs in the last competence state (Furukawa et al. 2000).

Macroglia in the Retina

Retinal glial cells are integral components of the neural retina, playing crucial roles in supporting neuronal function, maintaining homeostasis, and participating in the response to injury. These cells include two types of macroglia and one type of microglia, each with specific functions and developmental origins, contributing to the retina's complex architecture and functionality (Vecino et al. 2016). In some species with myelinated ganglion axons like in rabbits a fourth type, the oligodendrocytes, can be seen but for most mammals they are absent.

Müller Glia Cells

MGCs are the principal glial type in the retina making up 90% of macroglia in the retina. They extend radially across the entire thickness of the retina and form many cell contacts throughout all layers (A. Reichenbach et al. 1993). At the outer limiting membrane MGCs form apical microvilli that reach into the subretinal space between the photoreceptors (Uga and Smelser 1973). The basal process forms a thick endfoot that reaches the inner limiting membrane at the vitreal surface. This part of the cell is relatively stiff and rich in mechanosensitive ion channels. The remaining cell is elastic compared to the rather stiff neurons but tissue elasticity is mainly regulated by extracellular matrix components like collagen and proteoglycans in combination with water (Vecino et al. 2016). Throughout the nuclear layers lamellar processes extend between the neurons and envelop their cell bodies (Hama, Mizukawa, and Kosaka 1978). Due to their morphology MGCs provide a scaffold for migration and form columnar structures with radially aligned neurons they support (A. Reichenbach et al. 1993). This scaffold is essential for the architectural organisation and functional alignment of retinal neurons. Therefore, MGCs provide mechanical support and stability to the retinal architecture, which is crucial for the proper alignment of photoreceptors and the efficient transmission of light signals.

The role of MGCs is multifaceted. Besides some specialised tasks they share a lot of functions with other glial cells in the retina, mainly astrocytes. This seems redundant but for most tasks there is a preferred hierarchy in which the glial cells act and to a certain degree they can substitute for each other if necessary (Vecino et al. 2016).

MGCs are enrichened in crystallins that are also highly expressed in other ocular structures like the lens and the cornea (Delaye and Tardieu 1983; Krishnan et al. 2007). Crystallins like α-crystallin are expressed in MGCs and modulate the refractive index of the cell's soma (Moscona et al. 1985). This enables them to serve as light pipes across the retinal layers (Labin and Ribak 2010). Since MGCs span the whole width of the tissue they can directly transmit incoming light onto the photoreceptors outer segments reducing light scattering by the retinal neurons (Zayas-Santiago et al. 2018).

They are fundamentally involved in maintaining the retinal environment, regulating water homeostasis and the extracellular composition of ions and neurotransmitters which is crucial for neuronal signalling and photoreceptor function. In response to osmotic stress MGCs can not only rapidly regulate their own volumes to prevent swelling but also modulate their environment (Vogler et al. 2013). This can be mediated by water channels like Aqp4 and ion channels as the inwardly rectifying potassium channels Kir4.1 (Roesch et al. 2008). Blockage of Kir channels under hypoosmotic conditions resulted in the immediate swelling of MGCs indicating their importance in the regulation of homeostasis (Pannicke et al. 2004). Volume regulation in neuronal tissue is of great importance since small fluctuations directly influence neuron excitation. Neurotransmitter-mediated excitation of neurons causes transmembrane ion influx in the cell. Due to osmotic pressure this is accompanied with water influx, leading to swelling of neuron stomata, synapses and processes after intense excitation (Uckermann et al. 2004). If not compensated the swelling would cause a reduction of the extracellular space decreasing the synaptic distance between neurons, and therefore, increasing the excitation rate even further (Dudek, Obenaus, and Tasker 1990; Chebabo et al. 1995).

After light stimulation, K^+ concentration in the plexiform layers increases and needs to be decreased to prevent negative effects on excitability (Karwoski et al. 1985; A. Reichenbach et al. 1993). This can happen by two ways, diffusion in the extracellular space or by spatial buffering provided by MGCs (Andreas Reichenbach 1991). MGC endfeet are covert with ion channels and were shown to possess much more K^+ conductivity as the rest of the cell membranes (E. A. Newman 1987). A "cable" model

was suggested in which the cell takes up excess K^+ at the plexiform layers, and transports it towards the MGC endfeet where it gets disposed in the vitreal body which functions as a K^+ sink (Nilius and Reichenbach 1988). This prevents lateral diffusion-mediated excitation of adjacent neurons.

MGCs also contribute to the blood-retinal barrier, a selective barrier that isolates the neural retina from the systemic circulation (Tout et al. 1993).Together with astrocytes they are in direct contact with the retinal blood vessels and control the exchange of substances and thus protecting the retina from potential toxins and pathogens (Figure 9). Further, they are mediating metabolic exchange between the neurons and the vasculature providing retinal neurons with lactate and pyruvate while taking up waste products in turn (Tsacopoulos and Magistretti 1996). Glia are responsible for the main glucose uptake which is partly stored as glycogen. Despite the presence of oxygen the retinal cells tend to perform glycolysis instead of oxidative phosphorylation, termed aerobic glycolysis or Warburg effect (Casson et al. 2013). Therefore, they have a low oxygen consumption which could spare oxygen for the neurons deeper in the tissue (Winkler et al. 2000). Aerobic glycolysis is something usually seen in heavily proliferating or tumorous tissues (Ng et al. 2015). It was hypothesized that the aerobic glycolysis corresponds to the photoreceptor outer segment turnover that causes high biosynthesis requirements that extend efficient ATP production (Richard W. Young 1967; Poitry-Yamate, Poitry, and Tsacopoulos 1995; Vander Heiden, Cantley, and Thompson 2009).

Glucose uptake is also essential for neurotransmitter synthesis. Glutamate is the most abundant excitatory neurotransmitter in the retina, but it cannot be synthesised by neurons on their own. They require the glutamate precursor glutamine which is synthesised by adjacent glia cells. Inhibition of glutamine synthetase in the retina resulted in depletion of glutamate not only in MGCs. Surrounding bipolar and RGCs were depleted from glutamate as well showing their dependency on MGC-derived glutamine (Pow and Robinson 1994). MGCs are also involved in neurotransmitter recycling. They take up excess glutamate released by neurons via the excitatory amino acid transporter-1 (EAAT1) (Lehre, Davanger, and Danbolt 1997; Derouiche and Geiger 2019). Removal of glutamate from the extracellular fluid via transporter proteins is the only way to maintain a low nontoxic level of this neurotransmitter protecting neighbouring RGCs from excitotoxicity and enabling proper glutamate signalling (Kitano, Morgan, and Caprioli 1996; Kawasaki, Otori, and Barnstable 2000). Thereafter, glutamine synthetase converts the glutamate to glutamine which is transported back to neurons and can then be used again for glutamate synthesis (Danbolt 2001). Glutamine synthetase even enhances the uptake of excess glutamate from the extracellular space (Shaked, Ben-Dror, and Vardimon 2002).

MGCs are directly and indirectly involved in neuroprotection via a plethora of trophic factors. Expression of glial derived neurotrophic factor (GDNF) triggers signalling cascades after MGC receptor activation leading to expression of effectors like osteopontin (Del Río et al. 2011). This leads to an indirect MGC-mediated neuroprotective effect on photoreceptors (Frasson et al. 1999; C. Harada et al. 2003; Hauck, Toerne, and Ueffing 2014)

In response to hypoxia or injury MGCs produce the pigment epithelium derived factor (PEDF) early in gliosis. This serine proteinase inhibitor is an anti-angiogenic factor that counteracts elevated levels of vascular endothelial growth factor (VEGF) (Dawson et al. 1999; K. Park et al. 2011). PEDF is known for its anti-inflammatory effects and counteracts oxidative stress-mediated apoptosis and light damage, indicating its function as a survival promoting factor for several neurons (Cao et al. 2001; Cayouette, Barres, and Raff 2003; S. X. Zhang et al. 2006; Tsao et al. 2006). Especially RGC loss under ischemic or glaucomic conditions can be prohibited or diminished by PEDF signalling (Takita et al. 2003; Zhou et al. 2009; Unterlauft et al. 2012).

Neuroprotection of photoreceptors was also shown via bFGF, ciliary neurotrophic factor (CNTF) and BNDF signalling (Wahlin et al. 2000; Zack 2000). In another study, BNDF and bFGF showed elevated

survival of bipolar cells in dissociated primary explants. It was suggested that activation of a neurotrophin receptor on MGCs by BNDF might induce bFGF secretion since the receptor inhibition decreased the neuroprotective effects on bipolar cells by BNDF but not bFGF and inhibition of bFGF showed the same result after BNDF treatment (Wexler, Berkovich, and Nawy 1998).

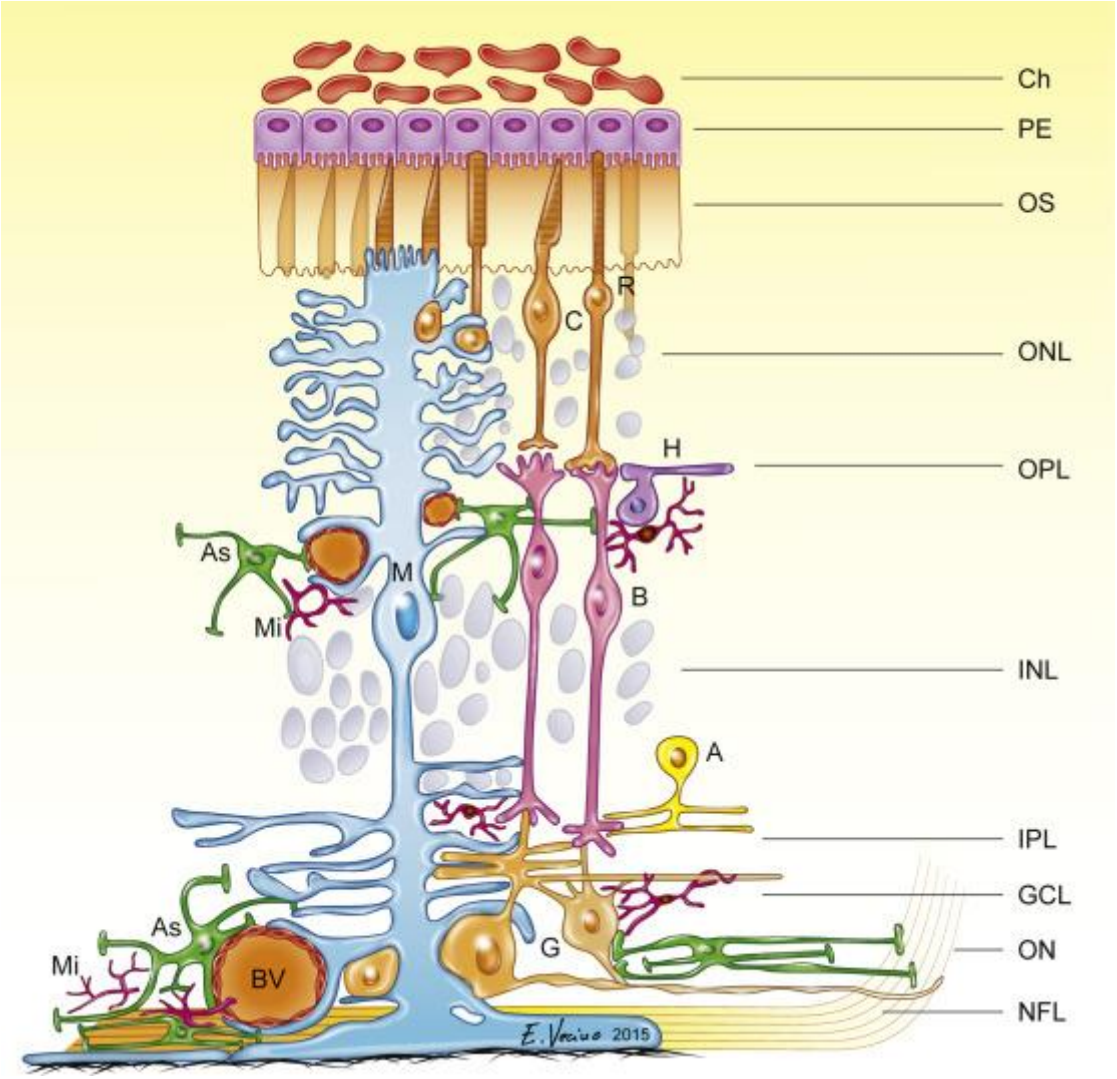


Figure 9: Glial cells in the neural retina. *Localisation of the three glial cells present in the in vivo retina. MGCs depicted in blue and are characterised by their radial morphology spanning the whole retina. They possess thick endfeet that are in contact with the basal lamina and microvilli on the apical side where they reach the photoreceptor outer segments. Retinal astrocytes, here shown in green, are mainly responsible for the blood-retina barrier and are located near blood vessels. Therefore, residing mainly in the ganglion cell layer on the basal side. Microglia shown in magenta are the main immune cells in the retina and distribute. BV: blood vessels; A: amacrine cells; As: astrocytes; B: bipolar cells; C: cones; G: ganglion cells; H: horizontal cells; M: Müller cells; Mi: microglia; R: rods; C: cones. Retinal layers: ON: optic nerve; NFL: nerve fibre layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; OS: outer segment layer; PE: pigment epithelium; Ch: choroid). (Image from Vecino et al. 2016)*

During retinogenesis MGCs arise from the same multipotent progenitors as the retinal neurons and their development is tightly controlled by a network of transcription factors and signalling pathways that guide their differentiation and subsequent maturation (Turner and Cepko 1987). The Notch signalling pathway was identified as a regulator of cell fate decisions such as self-renewal and cell survival during development (Austin et al. 1995; Bao and Cepko 1997). It was shown to be involved in

the differentiation of glia and neurons in the cortex and retina (Gaiano, Nye, and Fishell 2000; Furukawa et al. 2000; Morrison et al. 2000). Especially in late retinal development, Notch signalling plays a role in the differentiation of bipolar cells and MGCs. Intercellular signalling via Notch regulates the gliogenic switch that allows the differentiation of MGCs in the last competence state of retinal development. Even after the cell cycle exit Notch signalling needs to be maintained in MGCs for the stabilization of the glia fate and maturation process (Nelson et al. 2011). Notch1 and Hairy and enhancer-of-split homologues (Hes) family members are therefore found in mature MGCs (Furukawa et al. 2000). Hes1, Hes5 and Hey2 are downstream effector genes of Notch that repress the transcription of pro-neural genes (Riesenberg et al. 2018; Ohtsuka et al. 1999).

During retinal differentiation of mice Hes1 is expressed in the apical region where undifferentiated progenitors divide. Thereafter, its expression decreases with ongoing differentiation until it reaches very low levels around P10 before it gets expressed again in MGCs (Tomita et al. 1996). Hes1 is a known negative regulator of differentiation and antagonizes the expression of the proneural bHLH genes Mash1, Math1 and Atoh7 (Ishibashi et al. 1994; Takatsuka et al. 2004; Sasai et al. 1992). Conversely, inhibition of Hes1, for example by Hes6, leads to increased neuronal differentiation and increased expression of Mash1. Furthermore, Hes1-null mice exhibit premature neuronal differentiation (Bae et al. 2000; Ishibashi et al. 1995) Additionally, the forced expression of Rx, Notch or Hes1 in RPCs resulted in the upregulation of the MGC markers cellular retinaldehyde binding protein (CRALBP) and Cyclin D3 (Furukawa et al. 2000). Since upregulation of Rx expression leads to the expression of Notch and Hes1 it was suggested that Rx acts upstream of Notch which then regulates Hes1 (Furukawa et al. 2000). This is in accordance with the finding that mature MGCs maintain Rx expression (Yoshimoto et al. 2023).

Besides the expression of the transcription factors Sox9, Lhx2 and Rx members of the Nuclear Factor 1/I family were identified to be expressed in the late RPC pool that generates MGCs. The transcription factors NFIa, NFIb and NFIx regulate gliogenesis in the CNS and cell-cycle exit especially in late retinal cell differentiation (Deneen et al. 2006; Clark et al. 2019). Sox9 itself is not sufficient to initiate gliogenesis but it was shown to directly regulate the activation of NFIa, which is crucial for gliogenesis (P. Kang et al. 2012).

In embryonic spinal cord progenitors, NFIa and NFIb were sufficient to induce glia fate *in vivo*. On the other hand, NFI factors were not able to repress neurogenesis like the Hes family members (Deneen et al. 2006). Therefore, it could be possible that Notch does not directly activate the gliogenic switch but rather indirectly influence gliogenesis by mediating the amounts of progenitor cells (Androutsellis-Theotokis et al. 2006; Molofsky et al. 2012).

Epidermal growth factor receptor (EGFR)-mediated signalling is also associated with increased MGC differentiation. High amounts of TGF-α inhibit photoreceptor differentiation *in vivo* and increased the MGC fate decision (Lillien 1995). Normally, the expressed amount of receptor is the limiting factor for EGF signalling. In studies where additional EGF-R was introduced in RPCs *in vitro* the concentration of TGF-α required to shift the fate was reduced and *in vivo* experiments showed increased proportion of MGCs (Lillien 1995).

Little is known about MGC heterogeneity but transcriptome analyses indicated expression of some genes in only a subpopulation, something that was shown for astrocytes as well (Roesch et al. 2008; Bachoo et al. 2004). This is consistent with earlier findings showing expression of Vsx2 is restricted to some MGCs (Rowan and Cepko 2004). Besides this, the MGC expression profile overlaps strongly with the expression profile of mitotic RPCs (Blackshaw et al. 2004; Roesch et al. 2008). The cells are able to re-enter the cell cycle and show potential to dedifferentiate and produce neural cell types after injury in chick and rodents (Das et al. 2006; Fischer and Bongini 2010). The regenerative potential in humans

is limited and, therefore, MGCs and their response to injury is heavily studied (Pollak et al. 2013; Jayaram et al. 2014). Under normal pathologic conditions glia cells undergo reactive gliosis causing the upregulation of glial fibrillary acidic protein (GFAP) and proliferation to fill the gap between dead neurons, building glial scars, that can potentially inhibit neuronal regeneration and disrupt retinal function (Bringmann et al. 2006).

Retinal Astrocytes

Astrocytes are the most abundant cells in the brain (Molofsky et al. 2012). They are a heterogeneous population and different brain regions contain glia with specialised functions (Somjen 1988). Fibrous astrocytes are found in the white matter and exhibit the classical "star shape" with dense intermediate filaments in their processes. Protoplasmic astrocytes populate the grey matter, they have fewer intermediate filaments and are irregularly shaped (Barres 2003).

In the retina, astrocytes are the second type of macroglia. While some species like primates, pigs and minipigs have two morphologically distinct astrocyte types in the retina that connect either to blood vessels or RGCs , mice and rats only have one (Ogden 1978; Vecino et al. 2016). Even though some astrocytes exhibit an elongated morphologys retinal astrocytes still seem to resemble the heavily $GFAP^+$ fibrous astrocytes exhibiting mostly small somas and radial processes (Stone and Dreher 1987). Astrocytes and oligodendrocytes derive from two neural progenitor cell populations in the optic nerve (Eroglu and Barres 2010). Upon CNTF and LIF stimulation, $Pax2^+$ and $A2B5^+$ astrocyte precursor cells (APCs) give rise to type 1 astrocytes. (Huaiyu Mi and Barres 1999; H. Mi, Haeberle, and Barres 2001). $NG2^+$ precursors give rise to oligodendrocytes as a default but upon isolation their differentiation into type 2 astrocytes can be stimulated by CNTF treatment (Small, Riddle, and Noble 1987). It was long argued if such a shared oligodendrocyte-type-2 astrocyte (O-2A) progenitor exists. As following attempts to isolate such cells from *in vivo* conditions failed, the O-2A progenitor was considered as an *in vitro* artefact (Skoff 1990; Fulton, Burne, and Raff 1991). Lineage tracing experiments indicated that protoplasmic astrocytes can be generated from a distinct subpopulation of $NG2^+$ cells in embryonic brain and spinal cord but they do not originate from a common bipotential progenitor (X. Zhu, Bergles, and Nishiyama 2008; X. Zhu et al. 2011). Post-mitotically, they are committed to generate oligodendrocytes only (Rivers et al. 2008; S. H. Kang et al. 2010). Nevertheless, both cell types, the oligodendrocytes and the type 2 astrocytes, remain excluded from the retina (Ffrench-Constant et al. 1988).

Angiogenesis extends the early vasculature built during embryogenesis (Risau and Flamme 1995). In contrast to MGCs that span the whole retina astrocytes are restricted to the vascularised areas of the retina (Figure 9). The vascular plexus is a laminar network containing arterioles and venules that are restricted to the nerve fibre layer and ganglion cell layer (R F Gariano, Iruela-Arispe, and Hendrickson 1994). Additionally, capillary beds form, one at the inner border of the inner nuclear layer and another capillary plexus is formed at the outer border of the inner nuclear layer shortly thereafter. The outer retina remains avascular (Provis 2001). Because of this, photoreceptors consume oxygen mostly from the choriocapillaris (Yu and Cringle 2001). The blood vessels form in an inner-to-outer-retina-manner and expand radially from the optic nerve head into the periphery by angiogenic sprouting of pre-existing vessels (Ray F Gariano 2003). In species, where vascularisation is sparse, the astrocytes are also low in numbers and retinae without vascularisation like in rabbit or guinea pig lack astrocytes completely (Schnitzer 1988; Yu and Cringle 2001). This is also true for avascular regions like the perifovea in primates (Henkind et al. 1975; Engerman 1976).

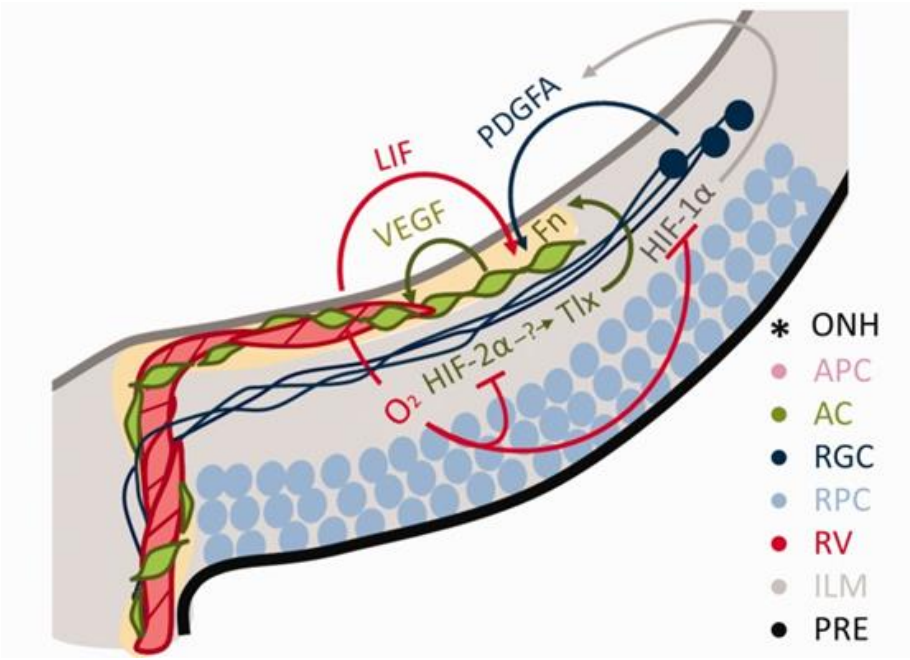


Figure 10: Retinal astrocyte-mediated angiogenesis of the retinal nerve fibre layer. *RPCs sense hypoxic conditions and express HIF-1α. This leads to the expression of PDGFA that acts via paracrine signalling on astrocytes migrating into the retina along the RGC axons. After activation of Tlx astrocytes assemble fibronectin and secrete VEGF. Vascular endothelial cells migrate on the fibronectin guided by VEGF signalling and counteract the hypoxic conditions. (Image adapted from Tao and Zhang 2014)*

Retinal astrocytes emerge from a mixture of APCs and immature astrocytes which migrate into the retina via the optic stalk prior to retinal angiogenesis (T. Watanabe and Raff 1988; Ling, Mitrofanis, and Stone 1989). APCs derive from the optic disk, a specialised progenitor region that encloses the optic nerve head like a cuff at the border to the retina (Dakubo et al. 2003; Morcillo et al. 2006). Gene expression profiles of the optic stalk and ventral retina overlap in this small ring defining a distinct population of cells between the tissues (Paisley and Kay 2021). Spindle shaped mesenchymal precursor cells that differentiate into endothelial cells proliferate and spread from the optic disc following the astrocytes at a distance of a few hundred microns as they spread in the nerve fibre layer (S. Hughes, Yang, and Chan-Ling 2000; Provis 2001). RGC axons provide migration guidance and cues via short range paracrine platelet-derived growth factor (PDGFA) signalling (Mudhar et al. 1993; Fruttiger et al. 1996). While the immature astrocytes exhibit strong proliferative capacity, the APCs possess high mobility. Three phases of astrocyte maturation can be distinguished: ACPs that are $Pax2^+$, $Vimentin^+$ and $GFAP^-$, immature astrocytes that are $Pax2^+$, $vimentin^+$ and $GFAP^+$ and mature astrocytes in the retina that are $Pax2^-$, $Vimentin^-$ and $GFAP^+$ (Chu, Hughes, and Chan-Ling 2001). Additionally, a small $Pax2^+$, $vimentin^-$ and $GFAP^+$ subpopulation represents mature perinatal astrocytes restricted to the optic nerve head.

Astrocytes release VEGF as a response to hypoxic conditions in the tissue prior to the vascularisation. VEGF signalling stimulates the following vascular endothelial cells and induces their proliferation, differentiation and migration (Tao and Zhang 2014). The endothelial cells in return secrete LIF which stimulate the astrocytes proliferation. VEGF is a common angiogenic factor and the hypoxia induced stimulation of endothelial cells by VEGF is a conserved mechanism among the retinae of many species (Chan-Ling, Gock, and Stone 1995). In accordance to the sequence of timely delayed capillary formation at the borders of the inner nuclear layer, VEGF expression was detected in this region shortly

prior to capillary formation (Stone et al. 1995). Since the signal emerges in the middle of the inner nuclear layer, it is more likely that this transient expression of VEDF is caused by MGCs, while astrocytes coordinate the hypoxia-induced response in the ganglion cell and nerve fibre layer. Hypoxia is accompanied with the expression of hypoxia inducible factor 1 (HIF-1) which is a regulator of oxygen homeostasis. In the ischemic retina, expression of HIF-1α showed spatial and temporal correlation with increased VEGF expression (Ozaki et al. 1999). It was later shown that signalling via HIF-1 subunit α by RPCs not only regulates expression of VEGF but also PDGFA expression by RGCs (Nakamura-Ishizu et al. 2012).

Glial cells are producers of extracellular matrix components like laminin, collagen and vitronectin (Vecino et al. 2016). During retinal angiogenesis retinal astrocytes produce fibronectin that is required as a substrate for the migrating endothelial cells following the VEGF signal (Jiang et al. 1994). Fibronectin matrix formation is regulated by the orphan nuclear receptor Tlx as demonstrated in Tlx null mice where extracellular assembly of fibronectin is severely altered.(Uemura et al. 2006). Tlx is expression by retinal astrocytes is upregulated under low oxygen conditiones but decreases over time when hypoxia is reduced by adjacent blood vessel formation indicating it could be a downstream element of HIF-1. Although the mechanism is not completely clear, Tlx is also a candidate thought to be involved in the regulation of start-stop behaviour in migrating astrocytes (Miyawaki et al. 2004). In Tlx mutant mice, the astrocytes mature faster and simultaneous fail to migrate beyond the central retina indicating a coupled mechanism that is linking their maturation and differentiation behaviour to the altered migration (Paisley and Kay 2021).

This hypoxia-mediated interplay between RGCs, retinal astrocytes and vascular endothelial cells enables the timely structured migration of retinal astrocytes and the angiogenesis of the retinal vasculature that supplies the tissue with nutrients and oxygen (Figure 10).

Even though several tasks of astrocytes in the brain are carried out by MGCs in the retina, they still contribute to maintain neuronal function. They ensheath axons, are involved in the formation blood-retina barrier, regulate water homeostasis via aquaporins, are involved in waste clearance, potassium buffering and calcium signalling. They secrete trophic factors, are involved in glucose metabolism and provide glutamine just like MGCs (Fields and Stevens-Graham 2002; Allaman, Bélanger, and Magistretti 2011; Tao and Zhang 2014; Vecino et al. 2016). Therefore, most of the glia marker proteins that are directly associated with their characteristic functions overlap. This holds also true for many signalling pathways that further underline the similarities between glia in the CNS. In the brain for example, just like in the retina, the glia versus neuron fate decision is mediated by bHLH genes (Nieto et al. 2001; Ross, Greenberg, and Stiles 2003). BMP2 signalling upregulates several negative bHLH genes in telencephalic neural precursors *in vitro*. This leads to inhibition of proneural bHLH genes like Mash1 and neurogenin causing increased astrocyte differentiation (Nakashima et al. 2001). The glia fate was long considered to be the default state in these tissues since the absence of proneural bHLH factors alone was enough to stimulate gliogenesis (Doetsch 2003). Identification of retinal astrocytes is, therefore, mainly accomplished by detection of immunostaining for GFAP as it is not expressed in healthy MGCs and only gets upregulated upon injury. Mature MGCs on the other hand can be distinguished from astrocytes by the expression of CRALBP, which is only transiently expressed in astrocytes during development but vanishes after postnatal week two in mice (Johnson et al. 1997).

Besides the macroglia there is one additional glial cell type in the retina the microglia (Vecino et al. 2016). They are the resident immune cells of the retina and originate from macrophage precursors in the yolk sac (Ginhoux et al. 2010; Schulz et al. 2012). They migrate into the retina during development and patrol the retinal environment, ready to respond to injury or disease (Carson et al. 2006). Their function is critical for phagocytosing debris, dead cells, and pathogens and they play a key role in the

inflammatory responses of the retina and modulation of the environment in response to stress or damage.

In summary, the development and function of retinal glial cells are central to the health and function of the retina. Through their support of neuronal structure and function, regulation of the extracellular environment, and roles in injury response, these cells ensure that the retina can perform its essential function of capturing and processing visual information. This complex interplay of development, maintenance, and repair highlights the integral role of glial cells in the visual system.

3D Cell Culturing Techniques

Mice have successfully been used as a retina model for decades. The murine retina has rod and cone photoreceptors but since mice are nocturnal animals and have comparatively more rod photoreceptors whereas cones make up only about 3% (Carter-Dawson and Lavail 1979; Jeon, Strettoi, and Masland 1998). Two types of cones have been identified in rodents that are, depending on the species, uniformly scattered or distributed on complementary halves of the retina (Szél et al. 1992; 1993; 1994). Additionally, in contrast to mice, primates like humans have a so-called fovea. This specialised region is a small pit that contains only cone photoreceptors at the highest density in the retina (Curcio et al. 1990). Thus, creating the region of highest acuity that is required for reading or driving. The fovea is located in the central region of the macular where sharp colour vision is possible and if damaged causes visual impairment (Remington and Goodwin 2004). In this region the ganglion cells are forming two or more layers in the ganglion cell layer to transmit all the information acquired in this receptor dense region. The amount of ganglion cells is even further increased in the fovea leading to a ratio of 2,6 ganglion cells per cone (Ahmad et al. 2003).

These examples show the differences that need to be addressed when transferring a hypothesis from one model system to another (van Beest et al. 2021). Basic research or medical research on the fovea cannot be translated into mice because of the absence of the structure. These differences between species demonstrate that there is often a trade off in animal models between the availability of the required tissue and the degree to which the model resembles the original (de Magalhães 2015). Simple model organisms like the zebrafish can be maintained at low cost but in high numbers compared to macaques that possess a fovea. Together with the ethical concerns tied to animal models these are major downsides of animal models.

2D culture models on the other hand are highly simplified and lack systemic context. They deprive the cultured cells of many stimuli that influence their growth, migration, differentiation or even survival (Kapałczyńska et al. 2018). These include topographic information, stiffness of the surrounding environment, cell-cell contact-mediated signals from other cell types as well as secreted factors (Engler et al. 2006; Kilian et al. 2010; Gilbert et al. 2010). However, some of these problems can be addressed like the modification of the growth substrate by coating with extracellular matrix proteins or the addition of soluble factors to the culture medium. Nevertheless, these actions substitute only a small fraction of the *in vivo* environment the cells lack in culture (Baker and Chen 2012).

The adoption of 3D cell culture techniques like organoids represents a significant advancement in research. It offers new insights into complex biological processes and disease mechanisms that were previously studied with the limitations of 2D cultures.

Organoid Technologies

Organoids are small simplified versions of an organ produced *in vitro* that resemble the micro-anatomy of a tissue (Pașca et al. 2022). These structures are derived from stem cells including ESCs or induced pluripotent stem cells (iPSCs) and are capable of self-organising into layered structures that mimic the architecture and function of organs (Rossi, Manfrin, and Lutolf 2018). Therefore, they resemble more physiologically relevant and complex systems for studying human tissues. Over the last years an increasing number of protocols emerged and the organs that can be modelled include, amongst others, liver, gut, lung, pancreas and prostate but also parts of the CNS like the brain and retina (Karthaus et al. 2014; Dye et al. 2015; Drost et al. 2016; Nikolić et al. 2017; Qian, Song, and Ming 2019; Hofer and Lutolf 2021).

The retina has been a major topic in organoid research since 2011 when optic vesicle-like structures with marker expression for RPCs and photoreceptors were firstly generated (Meyer et al. 2011). ROs are self-organising 3D structures derived from pluripotent stem cells that mimic the composition and architecture of the retina. The cells recapitulate the *in vivo* development, and therefore, depend on the same signalling cues including spatial gene expression, physical rearrangement, fate specification and cell sorting (Kratochvil et al. 2019). The methods to generate retinal tissue rely mostly on extra cellular matrix compounds that provide support and signalling cues combined with soluble factors like growth factors or small molecule inhibitors (Eiraku et al. 2011; J. Zhu and Lamba 2018; Z. Zhao et al. 2022). These approaches have revolutionized the study of retinal development and diseases by providing a model that recapitulates the key aspects of the human retina, including the formation of distinct neural layers, the generation of functional photoreceptors and the differentiation of MGCs (X. Zhong et al. 2014; Völkner et al. 2021). Some even described the generation of optic cups in an *in vivo* setup without extraocular tissue signalling cues (Eiraku et al. 2011; Nakano et al. 2012).

But the system has its limitations. Human ROs take a long time to reach a state where all cell types are present and mature, easily exceeding one hundred days of cultivation. Mouse-derived ROs, on the other hand, develop much faster but still lack the complexity of the *in vivo* tissue regarding cell numbers, level of maturation and cultivation duration (Z. Zhao et al. 2022). Continuously growing organoids share the problem of inhomogeneous nutrient distribution. Cell culture medium contains all necessary ingredients to support cultivation *in vitro* but it acts via diffusion in these systems. Depending on the size of 3D aggregates this can lead to a nutrient deficit in the central part of the organoid subsequently resulting in a so-called necrotic core. Therefore, the lack of vascularisation in these models is an issue that need to be addressed (Qian, Song, and Ming 2019). Another problem is that some cells like retinal astrocytes or microglia are not generated at their final destination and migrate during development into the tissue they later reside in (Vecino et al. 2016). In RO cultures the adjacent structures like the optic stalk are absent and therefore lack these cells and other feedback mechanisms. Approaches to tackle this, include mixed cultures of different cell types.

Assembloids, for example, are an extension of organoid technology, where different types of organoids are fused together or organoids and additional cell types are co-cultured or assembled (Pașca et al. 2022). In these models two or more separately generated specialised tissues are assembled to study potential interactions or boundary formation (H. Koike et al. 2019; Andersen et al. 2020). In the context of the retina, assembloids were already used to explore axonal outgrowth of RGCs into other parts of the central nervous system. Fused cultivation of ROs with cortical organoids caused neurons of retinal origin to grow in a directed manner towards the adjacent cortical tissue creating an optic nerve-like structure (Fligor et al. 2021; Fernando et al. 2022).

These 3D models recreate the spatial and cellular complexity of tissues more accurately, facilitating a deeper understanding of tissue development, function, and pathology. Even though problems with reproducibility emerge in increasingly complex cultivation setups, the benefits clearly overweight as they bridge the gap between traditional cell cultures and animal models.

Aim

ROs can be differentiated from ESCs or iPSCs and by a variety of differentiation protocols (Yuan et al. 2022). Most of them rely on the signalling cues of the extracellular matrix compound Matrigel in combination with growth factors or small molecule inhibitors. The induction of retinal fate in stem cells leads to the expression of the EFTFs that initiate the intrinsic program for retinal differentiation. From a common multipotent pool of RPCs, the six basic neuronal cell types and one glia cell type arise in a self-organised manner. One of the big breakthroughs in the field was the differentiation of optic cups from mESCs without the addition of extraocular tissue (Eiraku et al. 2011). But with increasingly complex model systems, the reliable reproducibility of such protocols remains challenging.

Aside from that, murine ROs only have limited cultivation time before they start to lose integrity. Starting on D25, the ROs display continuous loss of structure and thinning of the neuroepithelium. Cell numbers of the distinct neurons often diverge from the *in vivo* portions, and due to the restriction on cultivation time, remain immature and lack functionality. Since the differentiation of ROs relies completely on the accuracy with which the *in vivo* situation during development can be mimicked, it can be assumed that the maintenance and support of neuronal function relies on the presence of *in vivo* cues as well. Glia are an active part of the tissue that modulate the entire microenvironment via biophysical and biochemical environmental factors to maintain retinal function. Nevertheless, ROs mostly lack glia cells. MGCs, which are the last cell type to arise during retinal development, remain immature and in low numbers, while retinal astrocytes and microglia are completely absent due to their extraretinal origin. Therefore, the combined cultivation of retinal neurons with their supporting glia holds the potential to improve RO culture conditions.

Creating the necessary conditions for increased differentiation of MGCs directly during RO development would be the most beneficial way to maintain the integrity of the ROs and support an *in vivo*-like integration of this cell type. But simultaneously, other approaches will be followed that include the substitution with astrocytes. Compared to other glia cells, cortical astrocytes share the most similarities with MGCs, including water and ion homeostasis, especially K^+ regulation, synthesis of glutamine, neurotransmitter recycling, and neuroprotective signalling via trophic factors (Tao and Zhang 2014). Astrocytes are a physiologic cell type in the retina and have a designated niche in the retinal ganglion cell layer, which is beneficial for integration. Cell lines often exhibit changes in morphology and their gene expression profiles, which is why primary cells are advantageous when it comes to the functionality of a specialised cell type. Therefore, a protocol for the isolation of primary cortical astrocytes will be established (Schildge et al. 2013). Subsequently, immunostaining for specific marker proteins like GFAP and Aqp4 can be used to verify astrocyte identity and indicate their maturity.

To narrow down which mechanisms of action could improve RO growth, differentiation, or longevity, the experimental setups will follow two approaches, focusing on soluble factor-mediated and intercellular signalling. The secretion of neuroprotective factors could be beneficial for the retinal neurons, and supplementation with conditioned medium or cultivation in a shared environment is a good way to maintain RO integrity while enabling glia neuron communication. Additionally, a co-cultivation setup will be established where cell-cell contact-mediated signalling is possible. The dissociation of ROs followed by reaggregation with astrocytes will allow for direct contact between both cell types in a collective 3D microenvironment. To draw conclusions, the reaggregation behaviour of RO-derived cells and the composition of the generated aggregate need to be characterised first. Thereafter, aggregates that were enriched with astrocytes can be analysed for potential effects of the co-cultivation.

In the long term, the development of a model that combines neurogenic retinal cells and supporting glia cells in a shared culturing system is a promising approach to overcome the current limitations of organoids.

Material and Methods

All products were stored and handled as recommended by the manufacturer.

Table 1: Chemicals

Reagent	**Manufacturer** **Art. No.**
AGN193109	Sigma Aldrich SML-2034-5MG
β-Mercaptoethanol	Sigma Aldrich M6250
Blasticidin	InvivoGen Ant-bl-05
BSA (Bovine serum albumin)	Sigma Aldrich A4503
BSA Fraction V	Pan Biotech P06-1402020
DAPI (4',6-Diamidino-2-phenylindol Dihydrochlorid)	Carl Roth 6335.1
DMSO (Dimethylsulfoxid)	Sigma Aldrich D2650-5X5ML
DLL-4 (Recombinant Mouse)	R&D Systems 1389-D4-050/CF
DMEM (Dulbecco's Modified Eagle Medium)	Pan Biotech P04-03590
DMEM/F12 + GlutaMAX (1X)	Gibco 10565-018
Ethanol	Carl Roth 9065.2
FCS/FBS (Bovine Growth Serum Supplemented Calf)	GE Healthcare Hyclone SH30541.03
Jagged1 (Recombinant Mouse)	R&D Systems 10969-JG-050
Gelatin	Sigma Aldrich G6144
Geltrex	Thermo Fisher Scientific A14132
GMEM (Glasgow's Minimum Essential Medium)	Thermo Fisher Scientific 11570576
HBSS (Hanks' Balanced Salt Solution) w/o Ca and Mg	Thermo Fisher Scientific 12082739
HBSS with Ca and Mg	Thermo Fisher Scientific 15420614
KSR (KnockOut Serum Replacement)	Thermo Fisher Scientific 10828010

LIF (Leukemia inhibitory factor)	ESGRO ESG1107
Matrigel	Corning 356230, 354230, 356255
Mowiol	Merck 475904
N2-Supplements	Sigma Aldrich 17502048
NEAA (Non-Essential Amino Acids (100x))	Gibco 11140050
PanSera ES	Pan Biotech P30-2602
PFA (Paraformaldehyde)	Merck 30525-89-4
P/S (Penicillin/Streptomycin)	Gibco 15140-122
PD0325901	Sigma Aldrich PZ0162
PBS (Phosphate Buffered Saline) w/o Ca and Mg	Pan Biotech P04-36500
PBS+/+ with Ca and Mg	Corning 21-030-CVR
Poly-D-Lysine	Thermo Fisher Scientific 16021412
Pyruvate	Thermo Fisher Scientific 12539059
Retinoic acid (all trans)	Sigma Aldrich R2625
Sucrose	Carl Roth 9097.1
Surgipath	Leica FSC 22 Frozen Section Media
Taurine	Sigma Aldrich T8691
Tri-Sodium citrate (dihydrate)	Carl Roth 3580.1
Triton-X	Carl Roth 3051.1
Trypsin/EDTA	Gibco 15400-054
Trypsin 2.5 %	Thermo Fisher Scientific 11538876
Tween 20	Carl Roth 9127.1

Table 2: Media composition

Maintenance Medium (MM)
DMEM 15% PanSera 1% NEAA 1% P/S 0.1 mM β-Mercaptoethanol
Riken Maintenance Medium (Riken) GMEM 10% KSR 1% FCS 1% NEAA 1% Sodium Pyruvate 0.1 mM β-Mercaptoethanol
Retinal Differentation Medium (RDM) GMEM 5% KSR 1% NEAA 1% Sodium Pyruvate 0.1 mM β-Mercaptoethanol
Retinal Maturation Medium 1 (RMM1) DMEM/F12 1% N2 1% P/S
Retinal Maturation Medium 2 (RMM2) DMEM/F12 10% PanSera 1% N2 1% P/S
Astrocyte Maintenance Medium (Astro) DMEM 10% Heat Inactivated FCS 1% P/S

Table 3: Buffers and solutions

15% Sucrose Solution 150 mg Sucrose 10 ml PBS
30% Sucrose Solution 300 mg Sucrose 10 ml PBS

PBS-T 0.1% Triton-X in PBS
Sodium Citrate Buffer 10 mM Tri-Sodium citrate (dihydrate) in H_2O Adjust pH to 6 using 1M HCL Add 0.05% Tween 20

Table 4: Primary antibodies

Antigen	Host	Manufacturer	Product	Dilution
Aqp4	Rabbit	Alomone Labs	AQP-004	1:200
CRALBP	Mouse	Abcam	ab15051	1:200
CD44	Rat	Abcam	ab119348	1:200
GFAP	mouse	Stem Cell Technologies	60048.1	1:200
Islet1	Goat	R&D Systems	AF1837	1:200
Kir4.1	Rabbit	Alomone Labs	APC-035	1:200
NFIa	Rabbit	Abcam	ab228897	1:200
Otx2	Goat	R&D Systems	AF1979	1:200
P27 ^{kip1}	Rabbit	Abcam	Ab32034	1:200
pH3 (Ser10)	Rabbit	Sigma Aldrich	06-570	1:200
Sox2	Mouse	Abcam	ab171380	1:200
Sox9	Goat	R&D Systems	AF3075	1:200
Vimentin	Rabbit	Abcam	ab92547	1:200
Vsx2	Mouse	Santa Cruz	sc-374151	1:100

Table 5: Secondary antibodies and affinity proteins

Host	Antigen	Conjugate	Manufacturer	Product	Dilution
Donkey	Mouse	AlexaFluor 488	Jackson Immunoresearch	715-545-151	1:200
Donkey	Mouse	AlexaFluor 647	Invitrogen	A31571	1:200
Donkey	Rabbit	AlexaFluor 568	Invitrogen	A10042	1:200
Donkey	Rabbit	Cy5	Jackson Immunoresearch	711-175-152	1:200
Donkey	Goat	Cy3	Jackson Immunoresearch	705-166-147	1:200
Goat	Mouse	Cy3	Jackson Immunoresearch	115-165-146	1:200
Goat	Rat	AlexaFluor 568	Life technologies	A11077	1:200
Affinity Markers					
DAPI	DNA		Carl Roth	6335.1	1:1000
Phalloidin	Actin	AlexaFluor 488	Molecular Probes	A12379	1:200

Methods

All incubation steps were carried out at 37°C, 5% CO_2 and 95% humidity if not specified otherwise.

Stem Cell Maintenance

The transgenic mouse embryonic stem cell line EB9 Rx GFP was used. mESCs were passaged thrice a week. Cell culture flasks were coated with 0.1% Gelatin in 5 ml PBS for 15 min at room temperature (RT). The used medium was aspirated and the cells were rinsed twice with PBS before adding 200 µl of 0.25% Trypsin/EDTA in PBS. After 1 min at 37°C detachment was visually assessed and the cells were resuspended in prewarmed MM to stop the enzymatic reaction. The suspension was transferred into a 15 ml reaction tube and centrifuged for 5 min at 500 rpm. The supernatant was discarded and the pellet resuspended using 3 to 5 ml of prewarmed Riken Medium based on cell yield. Cell density was determined using a Neubauer chamber and reseeded in prewarmed Riken medium containing 2000 U/ml LIF, 1 µM Blasticidin and 20 µg/ml PD0325901. Supplements were added freshly each time. $4x10^5$ cells were seeded for a cultivation period of two days and $1x10^5$ cells for three days.

Differentiation of Retinal Organoids

The differentiation protocol of ROs from mESCs was based on the protocol of Eiraku et al. 2011.

On D0 mESCs were detached and seeded in a low adhesion well plate (Thermo Fisher Scientific #174925) for aggregate formation. Each well contained 3000 cells in 100 µl RDM supplemented with 0.1 µM AGN193109. One day after seeding (D1) extra cellular matrix compound was added at a final concentration of 2%. Since the compound required a protein concentration of at least 10 mg/ml it was necessary to vary between Matrigel and Geltrex depending on product availability. Higher protein concentrations were adjusted accordingly. After one week of cultivation the aggregates were assessed for retinal induction. By D7 aggregates showed signs of retinal differentiation like the formation of neuroepithelium and expression of the EFTF Rax indicated by GFP fluorescence. The aggregates were transferred into petri dished and further cultivated as floating cultures in RMM1. On D11 the RX-GFP^+ region formed optic vesicle-like structures that evaginated from the main aggregate. These were dissected manually using forceps. The resulting ROs were collected and transferred in a fresh petri dish containing RMM2 medium supplemented with 1 mM Taurine and 1 µM retinoic acid. Starting on D14 medium changes were conducted every two to three days using RMM2 supplemented with Taurine only.

Notch Ligand Treatment

As an attempt to increase glia generation in ROs soluble Notch ligands were added to the culture medium of 15 days old ROs. These were selected according to their GFP signal and quality of the neuroepithelium. On D15 ROs were transferred into a dish with fresh RMM2 supplemented with 1 mM

Taurine and 50 ng/ml of the soluble Notch ligands DLL4 and Jagged1. The ROs were fixed on D18 and afterwards prepared for sectioning.

Isolation and Maintenance of Cortical Astrocytes

The isolation of murine cortical astrocytes was conducted as described by Schildge et al. 2013.

Briefly four mouse pubs (C57 BL/6) were sacrificed between P0 and P4. Each brain was dissected performing a midline incision. The cranium was cut to access the brain which was transferred into HBSS on ice. Cerebellum and olfactory bulbs were removed and the brain divided into the two Hemispheres. Using forceps, the cortices were isolated from the brain and the meninges were removed entirely. The clean cortices were collected in a dish with fresh HBSS and cut into smaller pieces using a sharp blade. Cortical fragments were dissociated for 30 min at 37°C using a 0.25% Trypsin HBSS (w/o Ca^{2+} and Mg^{2+}) solution. After centrifuging for 5 min at 300 g the supernatant was discarded. The pellet was resuspended in 10 ml Astro MM and dissociated into single cells by forcefully pipetting 20 to 30 times. To discard possible tissue clumps the cell suspension was filtered using a 70 µm cell strainer (Thermo Fisher Scientific #11597522). Afterwards the volume was adjusted to 20 ml and completely transferred into one T75 flask that was previously coated for 1 h at 37°C using 50 µg/ml Poly-D-lysine in sterile water. To facilitate the adhering process the cells were incubated two days without moving. Staring on the second day after the isolation medium chances were done every two to three days.

After one week the astrocytes reached confluency microglia and oligodendrocyte precursor cells growing on top of the astrocyte monolayer were removed. Shaking the culture for 30 min at 180 rpm on a shaker removed most of the microglia. The supernatant containing the detached cells was discarded and 20 ml fresh medium added. Next the oligodendrocyte precursors were removed by shaking the flask for 6 h at 240 rpm. Remaining Oligodendrocyte precursors were detached by vigorously shaking the flask by hand. The supernatant was again discarded. The remaining astrocytes were enzymatically detached. Therefore, the flask was rinsed with PBS, treated with 1 ml of 0.25% Trypsin/EDTA in PBS and incubated at 37°C for 10 min. The cells were collected using 9 ml of Astro MM and centrifuged for 5 min at 500 rpm. The supernatant was discarded, the cell pellet resuspended in Astro MM and seeded into two freshly poly-D-lysine coated T75 flasks for further growth. The enriched astrocyte cultures were grown for additional two weeks until they reach the recommended degree of maturity and can be used for experiments.

Co-Cultivation Setup

First, astrocytes and ROs were cultivated in a shared environment but with minimal contact.

One T75 flask was rinsed with PBS, treated with 1 ml of 0.25% Trypsin/EDTA in PBS an incubated at 37°C for 10 min. The cells were collected using 9 ml of Astro MM and centrifuged for 5 min at 500 rpm. The supernatant was discarded and the cell pellet resuspended in 6 ml of Astro MM. 12-well plates were coated with Poly-D-lysine at 37°C for 1 h. After the coating solution was aspirated 500 µl of cell suspension were pipetted into each well of the plate. Volumes were adjusted to 2 ml per well and the plate set in the incubator for at least one day so the astrocytes could adhere and rest prior to the co-cultivation. Astrocytes need to be confluent by the time co-cultivation starts. The ROs were selected after 15 days of cultivation depending on their morphology. RO quality was defined by a roundish

shape and a well-defined epithelium containing bright Rx-GFP signal. If these requirements were met the medium on the astrocytes was exchanged for RMM2 with 1 mM Taurine and a single RO per well was added using a cut pipet tip. Every two to three days half of the medium was exchanged similar to regular RO cultivation. After a co-cultivation period of either three days (D15-18) or six days (D15-21) the ROs were fixed and prepared for cryosectioning.

Conditioned Medium

Conditioned medium (CM) consisted of Astro MM and was collected after three days of cultivating a confluent monolayer of matured astrocytes in a T75 flask. The supernatant was directly frozen and stored at -20°C. To test whether soluble factors secreted by the astrocytes influence RO differentiation they were cultivated for three or six days in 2 ml of a combined culture medium consisting of 1:1 CM and the regular RO growth medium RMM2. As a control the same experiment was conducted cultivating ROs in 2 ml unmodified Astro MM or RMM2 with heat inactivated Serum for at least three days.

Reaggregation of Retinal Organoids

For the direct co-cultivation of cortical astrocytes with retinal neurons in a combined microenvironment a new system was established. Approximately 15 ROs at D14 were transferred in a 1,5 ml reaction tube washed with PBS and treated for 10 min at 37°C using 500 µl of 0.25% Trypsin/EDTA in PBS. After the incubation the supernatant was discarded without stirring up the ROs. Then, 800 µl RMM2 were added to dissociated the ROs by pipetting them with a 1 ml pipet tip 30 times. The single cell solution was diluted and the cell number determined using a Neubauer chamber. To reaggregate the retinal neurons 5000 cells in 100 µl RMM2 w/o Taurine were seeded in each well of a low adhesion well plate. Growth was assessed and documented at D1, D4 and D7 after seeding. At D18 of cultivation aggregates were allocated to four petri dishes containing 6 ml of RMM2 supplemented with 1 mM Taurine each. From this point on half medium changes were performed every two to three days. The reaggregated organoids were cultivated for an additional 14 days after reaggregation and then fixed with 4% PFA.

Generation of Glia Neuron Assembloids

To generate a shared cultivation environment enabling direct contact between cell-types two conditions were investigated.

In the first approach the dissociated ROs were mixed with the astrocytes and seeded together in a low adhesion well plate to form an assembloid. Cortical astrocytes and ROs were dissociated into single cells using Trypsin/EDTA as described earlier. The seeding solution contained 5000 retinal cells and 3000 astrocytes in RMM2 w/o Taurine. After four days in the low adhesion well plate the assembloids were transferred into a petri dish with RMM2 containing 1 mM taurine.

For the second approach an aggregate of astrocytes was generated beforehand and retinal cells added thereafter leading to an assembloid with astrocytes in the centre and retinal cells in the periphery. First 3000 astrocytes were seeded in 100 µl Astro MM in a low adhesion well plate to form the "Glia core". Prior to adding the retinal neurons half of the Astro MM was discarded. Then 3000 retinal neurons (D15) in 100 µl of RMM2 w/o Taurine were added to envelop the astrocyte aggregate. After being transferred into petri dishes with RMM2 with 1 mM Taurine at D18 half medium changes were performed two to three times a week until they were fixed at D28 and prepared for cryosectioning. All assembloids were cultured identical to the reaggregated organoid control to ensure comparability.

Cryopreservation and Sectioning of Aggregates

ROs or assembloids were rinsed with PBS to remove residing medium and afterwards fixed with 4% PFA in PBS for 20 min at RT. The fixative was removed and the aggregate rinsed with PBS. To bridge the gap between the two osmolarities of the culture medium and the mounting compound necessary for cryosectioning samples were stepwise transferred into solutions with increasing concentrations of Sucrose. First the aggregates were put in 15% Sucrose solution for at least 1 h at RT and subsequently in 30% Sucrose solution over night at 4°C. Prior to the sectioning aggregates were collected using a cut pipet tip and gently mixed into Surgipath compound. After soaking for 1 h at RT the aggregates were placed inside the Cryotome (Leica CM3050) to freeze. The temperature settings were -18°C for the sample holder and -20°C for the surrounding chamber. The cross-sections have a thickness of 20 µm and were sequentially placed on Epredia™ SuperFrost Plus™ coverslips (Thermo Fisher Scientific # 10149870). The coverslips were incubated at a heating plate at 60°C for 2 h. Afterwards the sections were stored at -80°C until further use.

Immunohistochemistry

Samples were placed in a humid chamber with a lid to keep them in the dark and prevent evaporation during incubation times.

Cells were fixed for 10 min using 4% PFA in PBS. ROs and assembloids were already fixed prior to cryosectioning therefore this step was skipped. For antigen retrieval cryosections were placed in a holder and immersed in citrate buffer for 4 h at 60°C while slowly shaking. Samples were rinsed once with PBS and permeabilized for 10 min using PBS-T. Primary antibodies were added in a 1% BSA in PBS solution to minimalize unspecific binding. Cells were incubated for 2 h at RT and cryosections at 4°C over night. To prevent evaporation and facilitate evenly distribution of the antibody solution spacers were placed on both sides of the coverslip containing the cryosections and were thereafter covered with glass. Samples were washed three times for 5 min with PBS-T before adding the solution containing secondary antibodies and affinity proteins. Again, cells were incubated for 2 h at RT and cryosections at 4°C over night. After washing three times for 5 min with PBS-T samples were rinsed in H_2O to remove residing salt and were embedded in Mowiol. After drying over night in the dark at RT the samples were stored in the fridge until use.

Image Processing

The confocal laser scanning microscope (LSM) 800 by Zeiss was used for image acquisition via the Plan-Achromat 20x/ 0.8 air objective. ZEN software was used to stich tiles together and generate maximum intensity projections of z stacks. Further image processing was done using the ImageJ software.

Images were split into the acquired fluorescent channels. A manual threshold was applied on every protein staining and the DAPI channel as reference. The Rx-GFP channel was used as an unbiased template. First 50-pixel wide lines were drawn from the inside to the outside of the aggregate's epithelium (Figure 11 A). Per image 10-20 lines were used to measure the profile and average out small fluctuations. Lines were saved as region of interest (ROI) and later applied on the staining to measure protein localisation along the apico-basal axis. The measurements were further processed using Origin2023. The X values were normalized from 0 to 1. Using the feature *Average curves* with 100 datapoints. All measurements of one staining from the cryosections of one aggregate were averaged into data set. The resulting values of each Aggregate were averaged into one final curve for every experimental condition. Lastly the Y values were normalized to values between 0 and 1 (Figure 11 B and C).

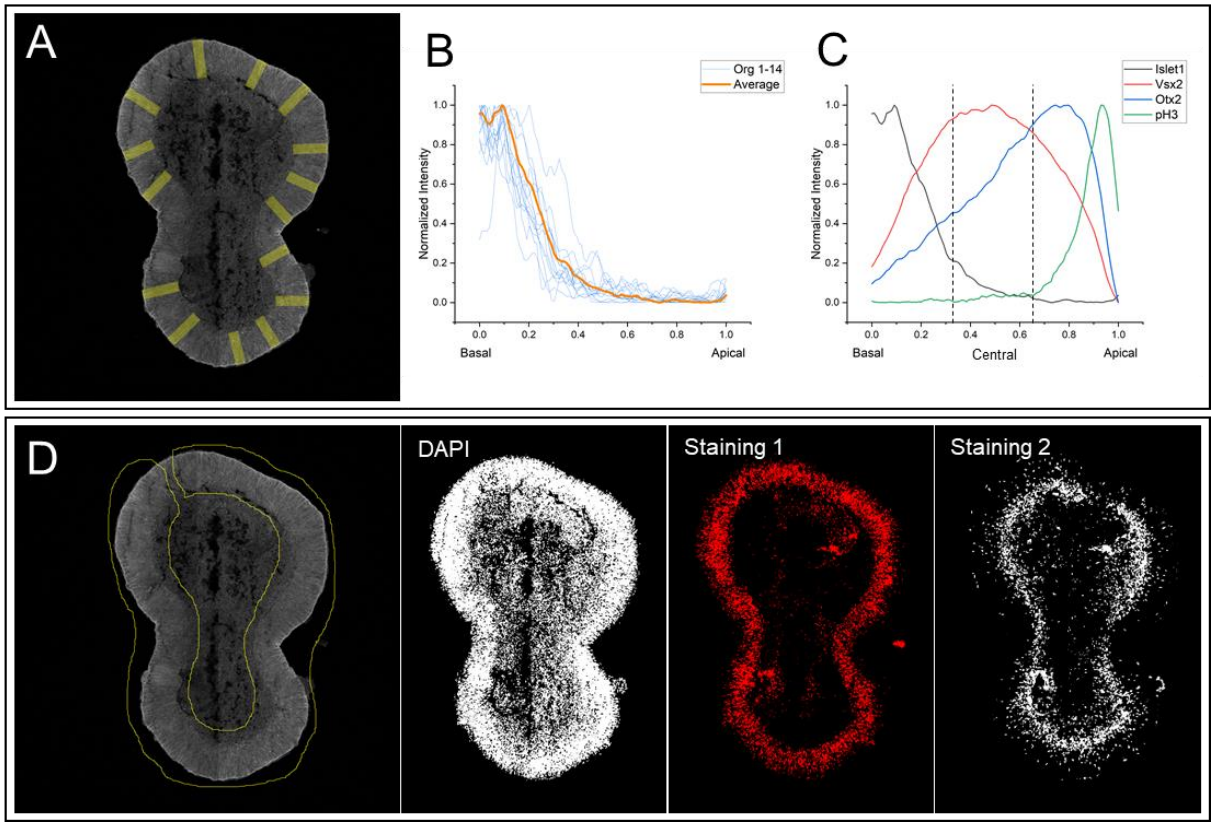


Figure 11: Analytic procedure for RO quantification. *Exemplary line measurements through the neuroepithelium of an 18-day old RO in the Rx-GFP channel (**A**). The resulting plot showing the measurements for the N=3, n= 14 ROs consisting of 3-5 sections with 10-20-line measurements each and the final line in orange representing the overall mean values to depict the localisation tendency (**B**). Epithelial localisation of the marker proteins Islet1, Vsx2, Otx2 and pH3 and their normalized grey value intensities. Distribution was classified in apical, central and basal localisation tendencies (**C**). The lower panel shows the region of interest defined in the RX-GFP channel for the relative cell number measurements of exemplarily staining 1 and 2 that can be calculated in relation to the DAPI channel or to each other (**D**).*

To determine the relative cell number positive for the stained protein the threshold of every channel was translated into a binary mask. The Rx-GFP channel was used to draw a line around the retinal epithelium excluding everything else from the ROI (Figure 11 D). Using the ImageJ plug in JACoP the number of pixels positive for the staining was calculated as a percentage of the DAPI positive mask. The result was the portion of immunoreactive nuclei in relation to all nuclei of the epithelium stained by DAPI. Additionally, co-localisation of two proteins of interest could be analysed by counting the positive cells for one staining as a portion to another staining.

Results

Differentiation of Retinal Organoids

The ROs were generated from a transgenic mESC line with GFP expression under the same promotor as Rax, which is one of the EFTF responsible for retinal determination of the early eye field. Therefore, the retinal induction could be assessed in the living organoid and monitored during the maturation process as a criterium for organoid quality. The stem cells were seeded in RDM with AGN in a low adhesion surrounding to generate embryoid body like aggregates (Figure 12 A). After one day cells compact into an aggregate that was embedded in the extra cellular matrix compound Matrigel or Geltrex. One week into the differentiation the aggregates formed Rx-GFP^{+} neuroepithelial structures on the outer border of the aggregate (Figure 12 B). This indicated successful retinal induction in these areas and mainly positive aggregates were transferred into petri dishes with maturation medium (RMM1) as floating cultures. Over the next four days retinal areas further developed into optic vesicle-like structures. The RX-GFP^{+} areas evaginated out of the main organoid to form smaller bean shaped vesicles (Figure 12 C). These vesicles were manually separated from the mother organoid by using forceps and the transferred in the second and last maturation medium (RMM2) supplemented with retinoic acid and taurine. Each of the dissected vesicles forms a new RO. Only healthy organoids were used for the experimental procedures characterised by a smooth surface, a bright epithelium in brightfield imaging and strong Rx-GFP signal.

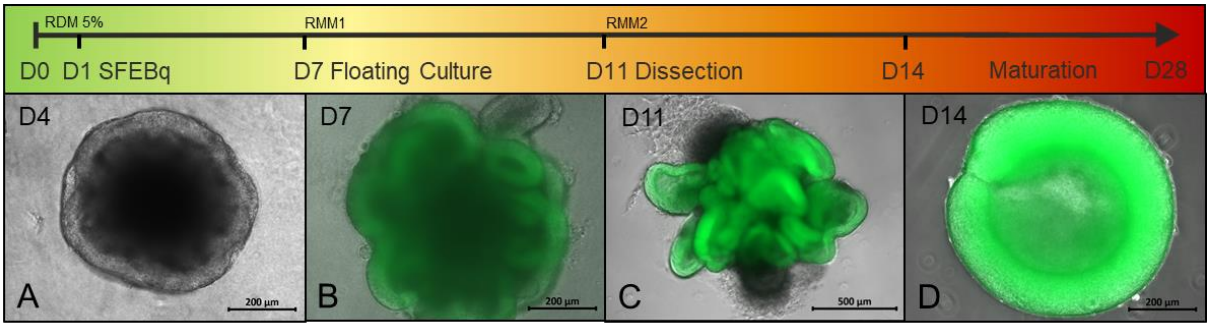


Figure 12: Timeline of the differentiation protocol for the generation of mESC-derived ROs. *Depicted are the stages of development starting with an embryoid body-like aggregate (**A**). After 7 days the aggregate shows signs of retinal induction leading to the expression of Rx-GFP in retinal regions (**B**). The regions start to grow out to form optic vesicle-like structures (**C**) that are dissected on day 11 to generate single roundish ROs that continue to mature in floating culture (**D**).*

Apico-Basal Polarity of the Retinal Neuroepithelium

To improve the general understanding of RO composition and its changes over time immunohistochemical staining of 20 µm thick cryosections were performed. The ROs were mounted as a whole and cut into thin cross-sections (Figure 13 A). Specific marker proteins were chosen to identify neuronal cell types each occupying a distinct localisation inside the neuroepithelium. This allows to translate the proximal-distal side in organoids to the apical-basal axis from the *in vivo* retina.

Magnification of the epithelium exemplifies the localisation for the staining of the marker proteins Vsx2 and Islet1 in the epithelium and displays distinct nuclei and their morphology (Figure 13 B and C). The expression of Rx-GFP indicated the retinal character of the tissue. The neuroepithelium can be clearly distinguished from remaining inner cells as seen in the DAPI channel. In comparison to the cells beneath the neuroepithelium the cells inside the epithelium show collective alignment. They have elongated nuclei that are oriented radially in the epithelium suggesting a polarization of the tissue (Figure 13 C).

The homeobox protein Vsx2 is one of the EFTFs necessary for the specification of the eye field. It is a marker for the first RPCs and is thereafter involved in bipolar cell specification. After terminal divisions, its expression is only maintained in bipolar cells and a subset of MGCs (I. S. C. Liu et al. 1994). In 18 and 21-day old ROs, the neuroepithelium is densely packed with $Vsx2^+$cells (Figure 13 E and F). They are uniformly distributed in this area but are restricted to the epithelium with a sharp border on the proximal side. After 28 days, the $Vsx2^+$ cells are not equally distributed across the epithelium and get restricted to the proximal side. Additionally, the LIM homeodomain factor Islet1 was stained to identify RGCs. The marker is widely used for ganglion cells specification even though it is also expressed in smaller subpopulations of bipolar, amacrine and horizontal cells (Bejarano-Escobar et al. 2015). During retinogenesis the RGCs are the earliest generated neurons and form the ganglion cell layer on the basal side of the retina. In ROs $Islet1^+$ cells can be found already at D15 (Data not shown) since their appearance precedes the differentiation of later born neurons which is in accordance to the conserved birth order of retinal neurons. In 18-day old ROs a large portion of cells is $Islet1^+$and already shows a specialised localisation at the proximal boundary of the neuroepithelium (Figure 13 panel D). The majority of $Islet1^+$ cells localises at the proximal border of the retinal epithelium while only sporadically $Islet1^+$ can be observed in more distal regions. There is a way bigger proportion of Islet1 expressing cells reaching into the inner part of the RO than cells spreading into the epithelium (Figure 13 B). This could indicate that the distal area of the organoid resembles the apical side in the retina.

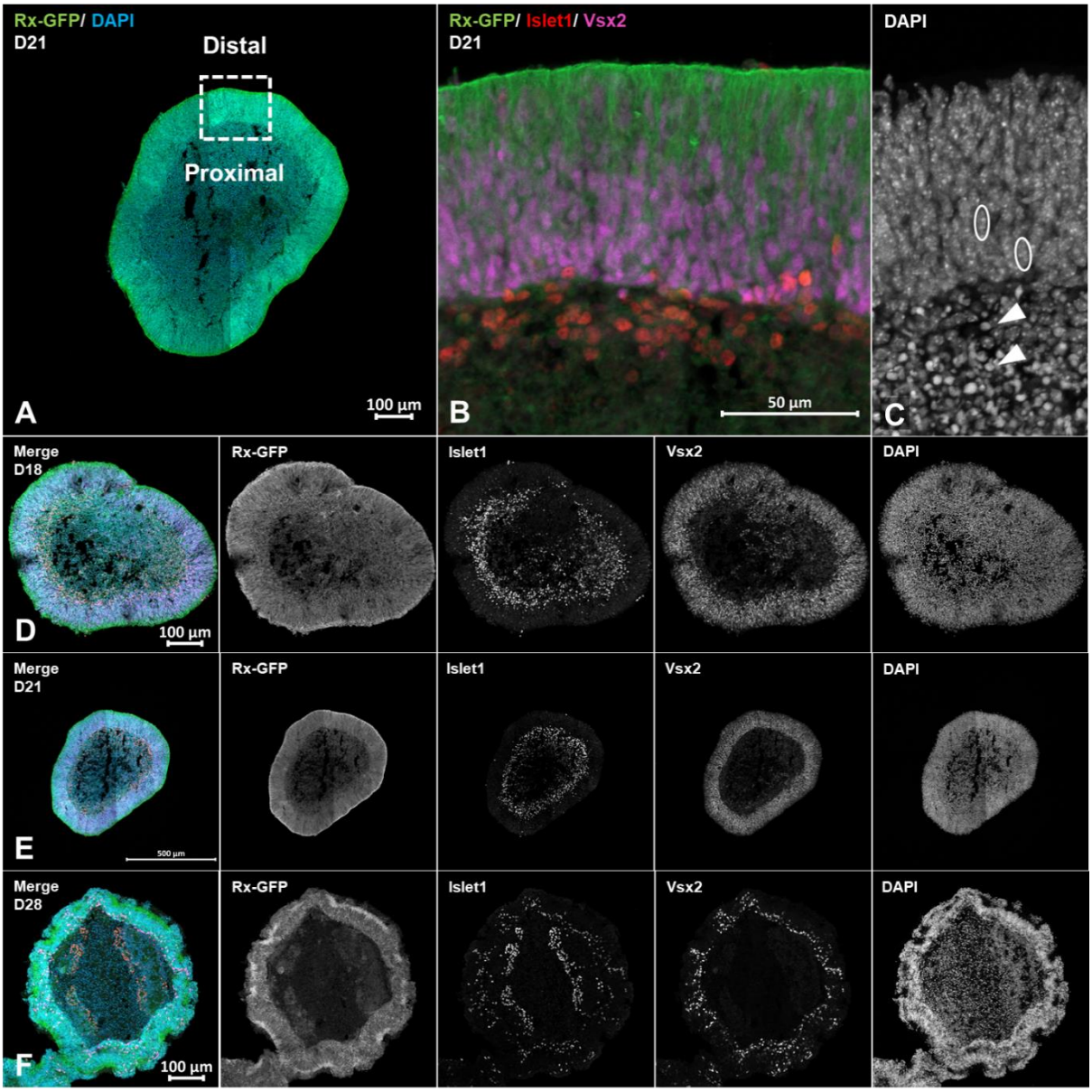


Figure 13: Localisation of interneurons in the neuroepithelium of 18, 21 and 28-day old ROs. *Cross-section of a 21-day old RO with highlighted exemplarily region of interest for magnification (**A**). Magnified view of the neuroepithelium of a RO with Islet1⁺ ganglion cells on the proximal side and Vsx2⁺ RPCs or bipolar cells distributed throughout the epithelium (**B**). The DAPI staining shows the different morphologies between nuclei inside the epithelium which tend to be elongated (**C** circles) or beneath the epithelium where they appear rounded (**C** arrows). ROs were fixed at D18, D21 and D28 to look into changes in localisation over time. The 28-days old ROs showed clear signs of structural loss of the epithelium and also contained fewer cells positive for both markers (**D, E, F**).*

To investigate whether the apico-basal organisation of the neuroepithelium in ROs is consistent for varying cell types, a marker for developing photoreceptors was used. These cells are naturally located at the apical side of the retina in the photoreceptor layer with their outer segments reaching into the RPE. Otx2 is involved in photoreceptor progenitor cell differentiation and remains expressed during development into matured photoreceptors (Nishida et al. 2003). Immunohistochemical staining of 18-day old RO cross-sections showed a broad distribution of $Otx2^+$ cells in the neuroepithelium. During photoreceptor differentiation in the retina and upon further maturation photoreceptors cluster at the apical side to form a dense layer. This behaviour is resembled in the RO development. On D21 a preference of the cells for the distal side of the epithelium was noticed that persists once established

(Figure 14 B and E). Even though the ROs start to show first signs of degeneration and loss of structure the general organisation of the cell types examined remains the same over time.

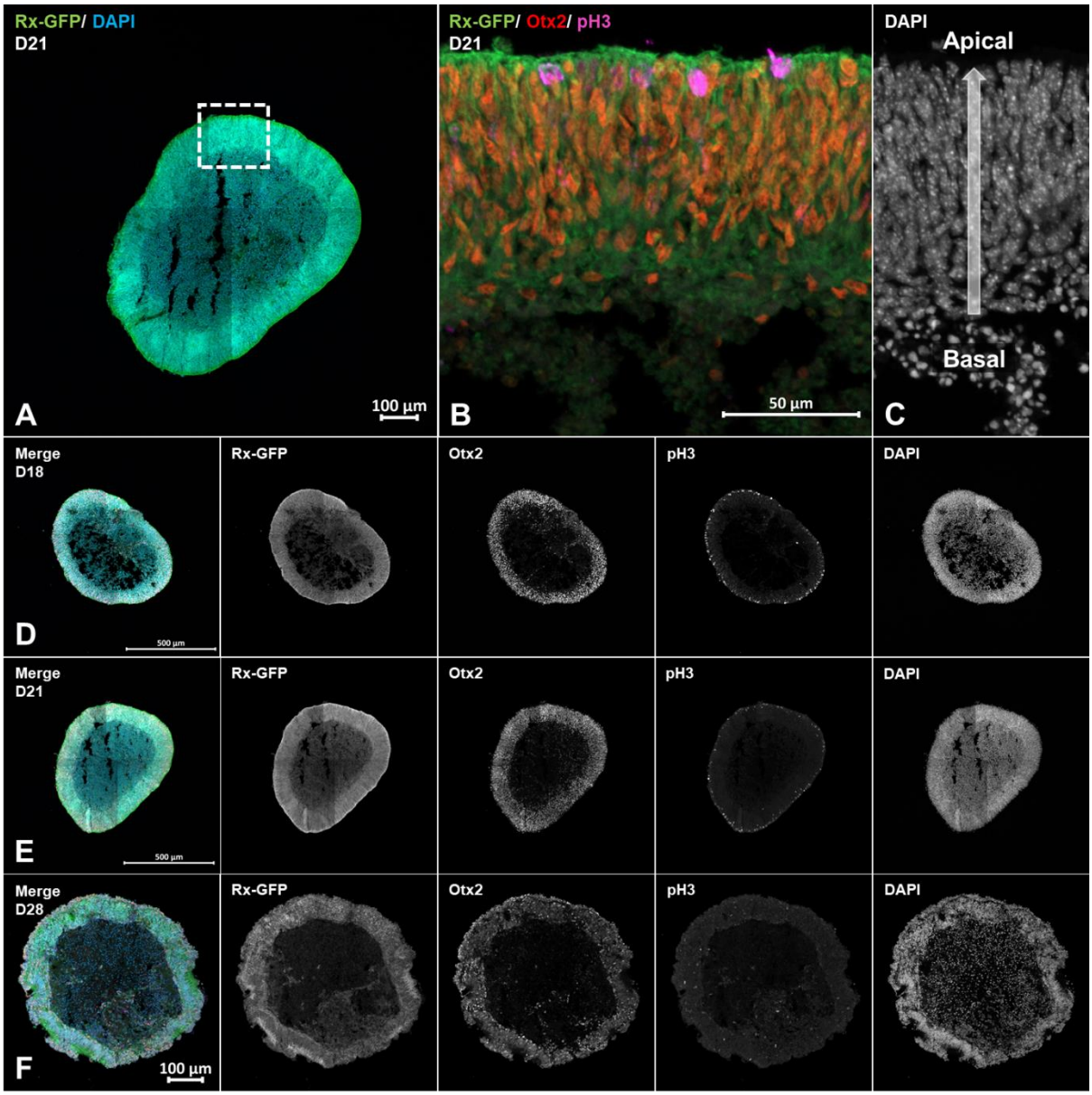


Figure 14: Localisation of photoreceptors and mitotic cells in the neuroepithelium of 18, 21 and 28-day old ROs. *Magnification of the neuroepithelium shows the localisation of Otx2⁺ cells distributed in the central part of the epithelium with a shift to the distal border while mitotic cells exhibit a strong affinity for the distal region (**A** and **B**). Due to the consistent similarities in the localisation of cell identities in proximal-distal orientation compared to the apical-basal structure of the retina these regions were further referred to as apical and basal as depicted in (**C**). 18 days old ROs show Otx2⁺ cells in the whole epithelium and a strong staining of pH3 that is restricted to the apical side (**D**). At day 21 the Otx2⁺ cells are still distributed in the epithelium but show a clear tendency towards the apical side. The number of mitotic pH3⁺ cells decreases but the limitation to the apical side persists (**B** and **E**). The ROs show signs of structural loss but the Otx2 cells were still present and exhibited brighter signal at the apical side. Mitotic cells decreased rapidly with RO age and the few remaining cells are no longer restricted to the apical side even though they maintain their affinity for it (**F**).*

Lastly, mitotic cells were stained using an antibody against the phosphorylated histone 3 serine 10. The phosphorylation starts at the early G2 phase and is a crucial step for the onset of mitosis (Crosio et al. 2002).

Notably all the cells that were reactive for this phosphorylation in ROs that were 18 and 21 days old were located at the distal side of the epithelium (Figure 14 B, D and E). In 28-day old ROs immunoreactive cells occur sparsely and were not as restricted to the distal border. This could be the result of the negative effects of aging in ROs like loss of structure. All marker proteins examined showed a distribution that resembles the organisation of the *in vivo* retina. Therefore, from now on the proximal side was defined as the basal side of the epithelium and the distal side will be referred to as the apical side (Figure 14 C).

Analysing the composition and developmental dynamics of RO neuroepithelial formation and maturation required quantification methods. To evaluate early and late cultivation timepoints of the ROs, but also for comparison with the *in vivo* development, two main criteria were addressed.

The first aspect is the localisation of retinal cell types in the neuroepithelium with a more detailed look on the apico-basal distribution and its time dependent rearrangements. These can be interpreted and compared to literature, give insights in the maturing process of cell types and whether the general dynamics of the retina are conserved in this *in vitro* model.

Therefore, micrographs were used to draw line measurements through the neuroepithelium and generate normalised plot profiles that represent the localisation tendency of a certain sell type along the apico-basal axis (Figure 11). Along this axis the middle third was defined as the central region in the epithelium. Changes in the distribution will be noticeable by the shift of the curves maximum or flattening of the curve.

The second aspect is the cell number of distinct neuronal cell types in the epithelium. The quantification was done by measuring the relative cell number as a percentage of the area covered compared to the DAPI channel. Since DAPI stained the nuclei of all cells regardless of their cellular identity this was used as a reference. To avoid unspecific background a threshold was applied and masks were created from all staining and the DAPI channel. Again, the region of interest that was measured was defined in the Rx-GFP channel displaying the outline of the neuroepithelium (Figure 11 D).

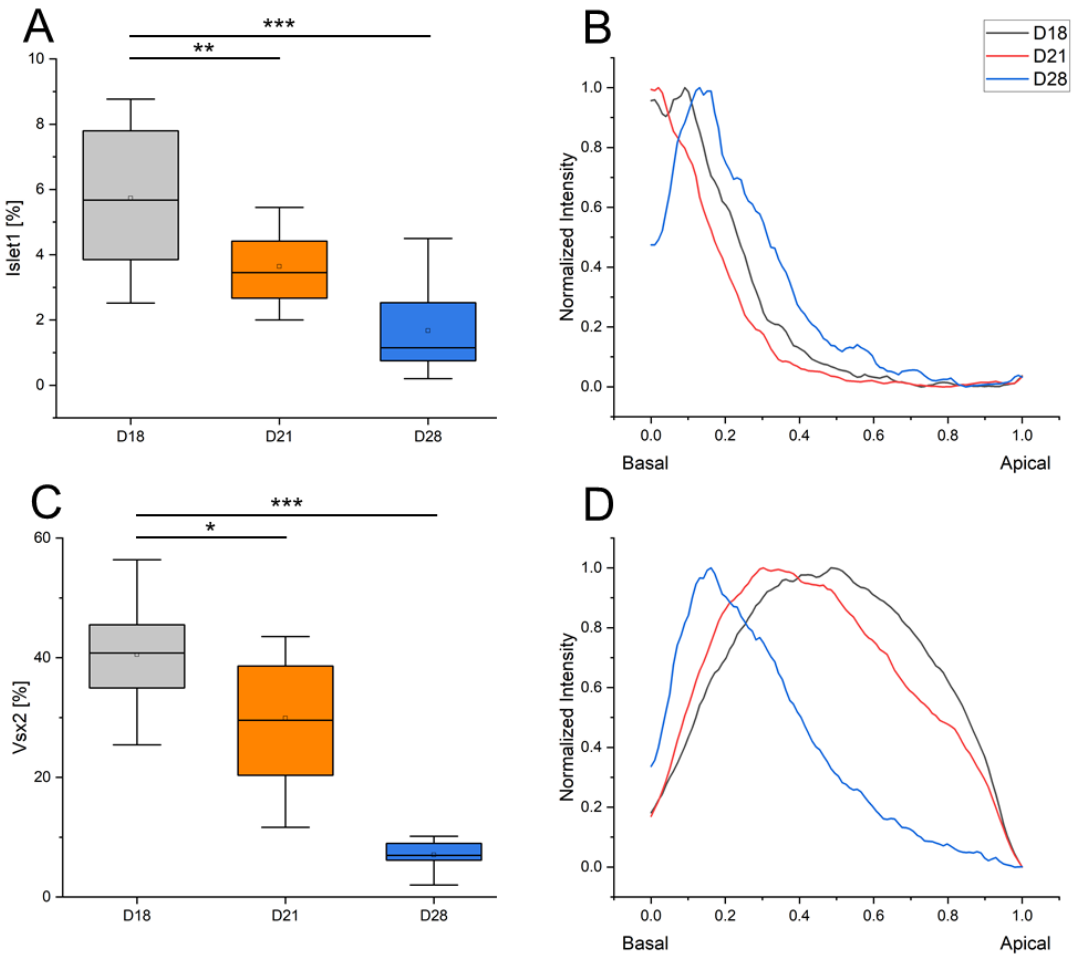


Figure 15: Interneuron distribution across the neuroepithelium of 18, 21 and 28-day old ROs. *Relative cell numbers of Islet1⁺ (**A**) and Vsx2⁺ (**C**) cells as proportion of all epithelial cells. Apico-basal localisation of Islet1⁺ (**B**) and Vsx2⁺ (**D**) interneurons over time. Sample sizes: A and C: D18 N=3, n=13; D21 N=3, n=15 D28 N=3, n=15. Two sample t-test: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.*

At D18, Islet1⁺ cells make up 5.73% ± 2.22% of the total neuroepithelial cells. This decreases significantly over time reaching 3.66% ± 1.15% on D21 and 1.67% ± 1.30% on D28 (Figure 15 A). Independent of the changes in relative cell numbers the apico-basal localisation remained restricted to the basal side for all timepoints analysed. All curves have their maximum on the basal side and show a strong fall of the curve in apical direction (Figure 15 B). In contrast to the Islet1⁺ cells that were present in low numbers, the Vsx2⁺ cells make up 40.52% ± 9.45% of the epithelium. After longer cultivation, the proportion of the cells decreased reaching 29.88% ± 10.70% on D21. Cell numbers further decrease drastically until D28 where only 7.05% ± 2.15% of the cells remain Vsx2 immunoreactive. This means there is a difference of over 30% in the portion of Vsx2⁺ cells between D18 and D28. The line measurements show that the localisation of Vsx2⁺ cells is mainly in the central area of the epithelium. On D21, the curve starts to shift towards the basal side but its maximum is still in the central region. This trend is consistent over time leading to the maximum to be shifted in the basal region even below the 0.2 mark. Additionally, the curve is steeper than the broader curves of the D18 and D21 ROs indicating a shift of the cell's location during this time (Figure 15 D).

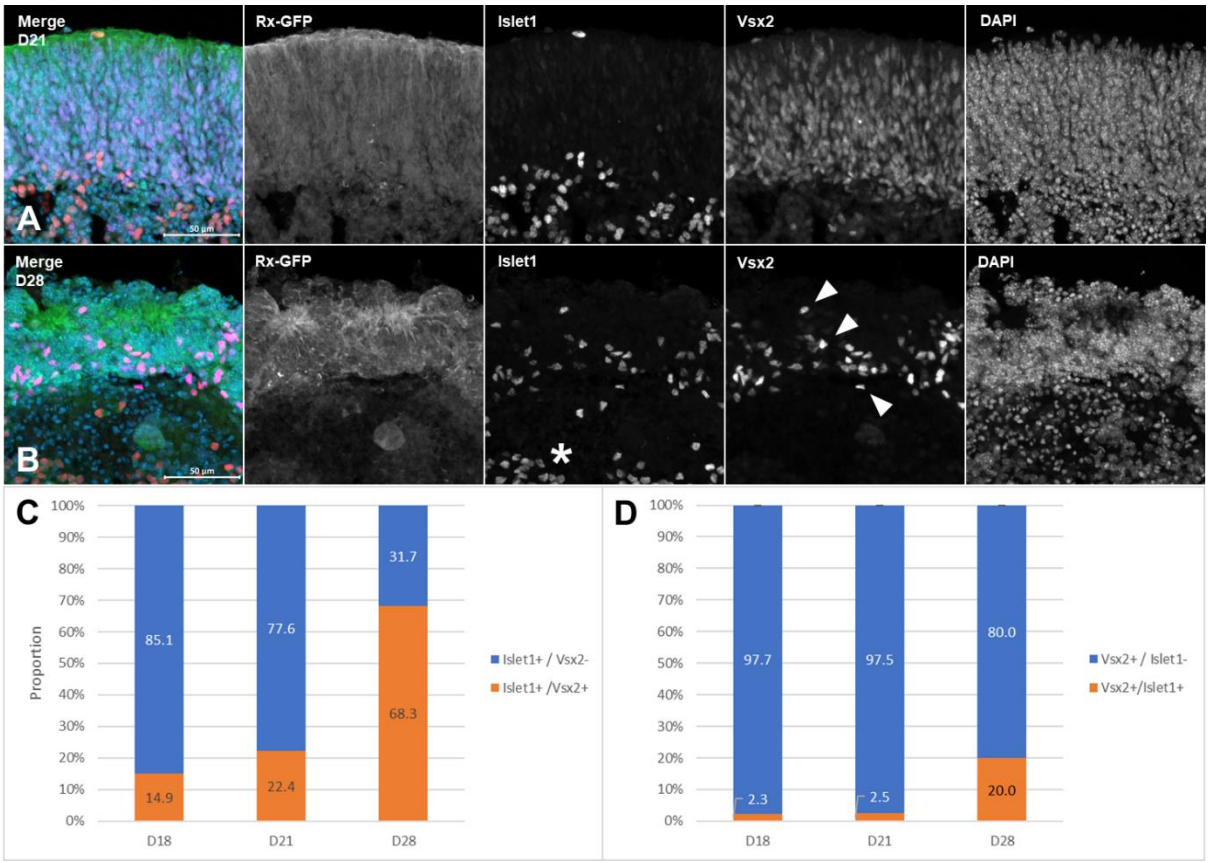


Figure 16: Identification of an Islet1⁺/Vsx2⁺ subpopulation of bipolar cells. *Microscopy images showing differences in the localisation of Islet1 (red) /Vsx2 (magenta) co-expressing cells (arrowheads) and Islet1⁺/Vsx2⁻ cells (asterisk) Panel **A** and **B**. Quantification of Islet1/Vsx2 co-expressing cells as proportion of Islet1⁺ or Vsx2⁺ cells in the epithelium (**C** and **D**). Sample sizes: D18 N=3, n=13; D21 N=3, n=15; D28 N=3, n=15.*

The micrographs showed that there are two distinct Islet1⁺ cell groups from which one is primarily found in late ROs. Islet1⁺ /Vsx2⁻ cells stain RGCs that remain located at the basal side of the retinal epithelium and can reach even areas beneath the epithelium. In 21-day old ROs the border between the broadly Vsx2⁺ epithelium and the Islet1⁺ cells is sharp (Figure 16 A). At D28, the border is dissolved and Islet1⁺ cells can be detected in the epithelium. These cells exhibit additional expression of Vsx2. Due to their localisation in the central epithelium and co-expression of Islet1 and Vsx2 it is most likely these cells represent a subgroup of late born bipolar cells. Meanwhile, a group of Islet1⁺ cells remain basally that do not show Vsx2 co-expression and was therefore categorized as RGCs (Figure 16 panel B). The Islet1⁺/Vsx2⁺ cells make up two thirds of the Islet1⁺ cells while accounting for 20% of the total Vsx2⁺ cells (Figure 16 C and D). Co-localisation measurements show increased numbers of Islet1⁺/Vsx2⁺ cells on D28 while in the meantime total numbers of Islet1⁺ and Vsx2⁺ cells in the epithelium decreased. This led to the conclusion that by D28 almost all Islet1⁺/Vsx2⁻ RGCs died. For the earlier timepoints D18 and D21 the share of Islet1⁺/Vsx2⁺ cells of the total epithelial cells were constant.

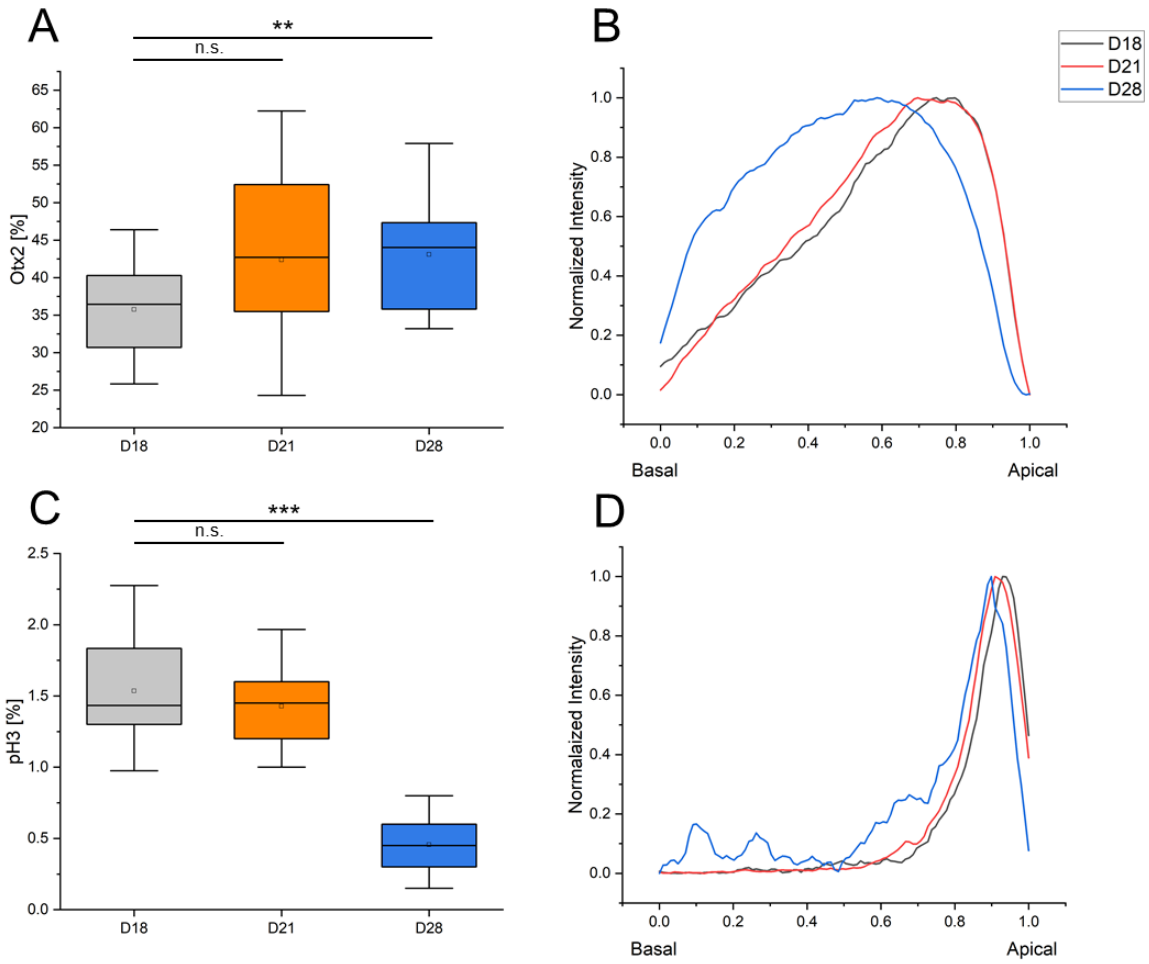


Figure 17: Photoreceptor and mitotic cells distribution across the neuroepithelium of 18, 21 and 28-day old ROs. *Relative cell numbers of Otx2⁺ (**A**) and pH3⁺ (**C**) cells as proportion of all epithelial cells. Apico-basal localisation of Otx2⁺ (**B**) and pH3⁺ mitotic cells (**D**) over time. Sample sizes: A and C: D18 N=3, n=14; D21 N=3, n=15; D28 N=3, n=15. Two sample t-test: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.*

For the Otx2⁺ photoreceptors cell numbers increased from 35.76% ± 6.48% at D18 to 43.11% ± 7.57% at D28. Even though relative cell numbers increased over the 3-day cultivation period from D18 to D21 reaching 42.39% ± 10.63% this tendency is not significant until D28 (Figure 17 A). The epithelial distribution further supports the similarity between the Otx2⁺ cell population in D18 and D21. Their distribution curve in the epithelium shows a similar localisation tendency displaying almost linear increase from basal to apical with its maximum at the apical side (Figure 17 B). The cells can be detected throughout the epithelium but are clearly accumulated at the apical side. This cellular organisation is lost in 28-day old ROs. Here Otx2⁺ cells are spread in the central area of the epithelium showing almost no preference for the apical side anymore (Figure 17 B).

Mitotic cells in the neuroepithelium make up about 1.54% ± 0.38% on D18 and 1.43% ± 0.28% on D21 showing close similarity of the mitotic activity during this differentiation period. This changes significantly when compared to ROs at D28 where mitotic activity was reduced by almost two thirds resulting in a portion of 0.46% ± 0.18% pH3⁺ cells (Figure 17 C). Regardless of the cell numbers mitotic cells were accumulated on the apical side. Beyond that pH3⁺ cells in D18 and D21 ROs exhibiting a strict limitation solely to the apical side. D28 ROs showed the same affinity for the apical localisation but with minor fluctuations caused by cells in the central and basal regions (Figure 17 D).

Identification of Glial Cells in Retinal Organoids

Since neurons are highly specialised they need supporting glial cells to modulate the environment and maintain neuronal function. However, glial cells emerge only late in development and in ROs they remain immature and in low numbers. Characterisation of glia cell occurrence is the first step to a better understanding of glia neuron interactions in ROs. Since marker proteins are closely related to the functions the cell serves in the tissue, glia cells share many common marker proteins making it hard to distinguish between them. This includes for example, water-and Ion-channels that are involved in the homeostasis of the surrounding tissue or structural proteins like the intermediate filament GFAP.

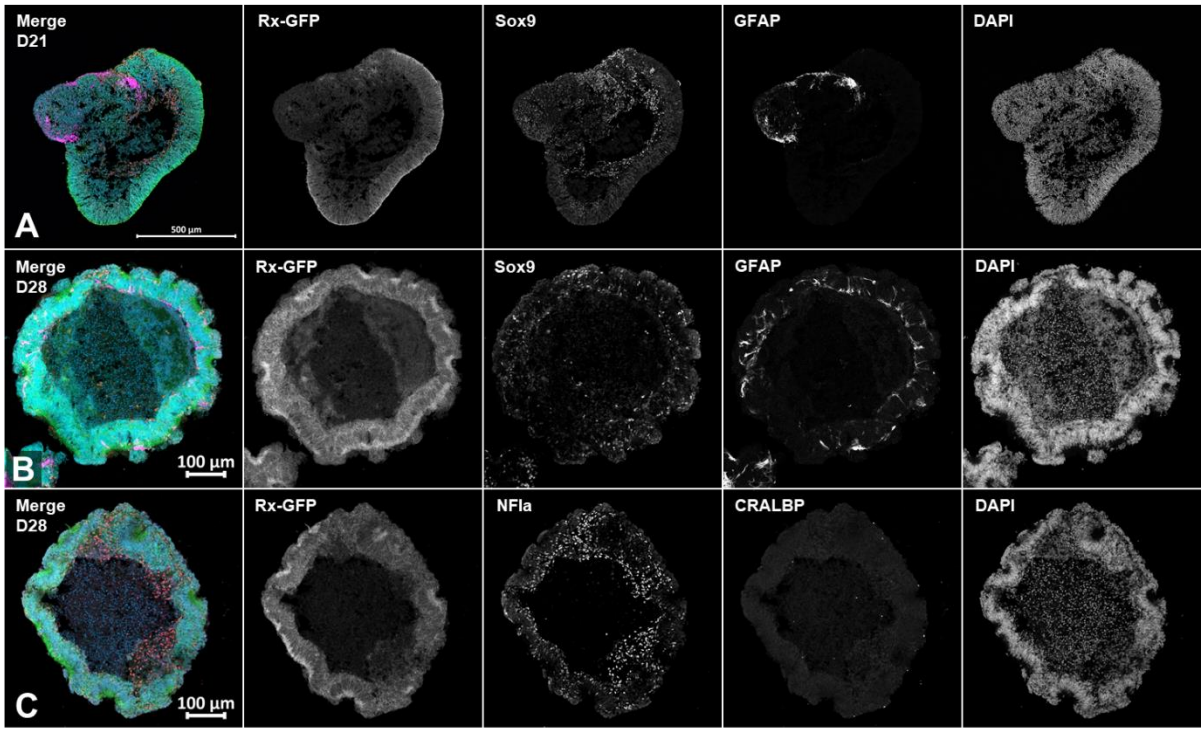


Figure 18: Occurrence of glial cells in ROs. *Glia cells expressing GFAP are restricted to the Rx-GFP$^-$ parts associated to ROs at D21 while Sox9$^+$ late RPC are present throughout the RO (**A**). Inside the neuroepithelium, GFAP was earliest detected on D28 and appeared only rarely. Exemplarily image showing the cell morphology of GFAP$^+$ cells with elongated processes (**B**). The MGC progenitor marker NFIa is expressed on D28 but still ROs were negative for the specific MGC marker CRALBP at all timepoints assessed (**C**).*

From all retinal glial cells MGCs are the only ones that are directly generated from the common multipotent RPC pool the retinal neurons also arise from. The other glia cells are generated externally and migrate into the retina during development. To detect the potential progenitor pool for MGC generation the transcription factors Sox9 and NFIa were stained. The transition from RPCs to MGCs is a fluent process where Sox9 and NFIa expression starts in late RPCs and is maintained in glial cells. Therefore, Sox9$^+$ and NFIa$^+$ cells in 21 and 28-day old ROs indicate either the presence of MGCs or their immediate progenitors (Figure 18). The appearance of GFAP$^+$ glial cells was firstly detected after 21 days of cultivation. In these cases, the glial cells were located in the Rx-GFP$^-$ region associated to the RO while the retinal epithelium was completely GFAP$^-$ (Figure 18 A). ROs containing GFAP$^+$ cells inside the neuroepithelium were detected on D28 at the earliest but occurred only rarely and with strongly varying portions of GFAP signal. These were ranging from a few clustered cells at one point of the RO

to a sparse distribution along the whole epithelium as seen in figure 18 B. Additional staining of CRALBP, which is a specific marker for MGCs, was performed to get more insights into the identity of the detected glia cells. CRALBP is involved in the visual cycle and is not expressed in other glial cells present in the retina. The protein is expressed in mature MGCs and the ROs were therefore stained at later timepoints of cultivation. On D28, no signal for CRALBP was detected (Figure 18 panel C). This could mean that o MGCs are present at all but also that the cells are not mature enough to express the protein. To avoid misinterpretation, ROs were further cultivated. But even after an extended cultivation time until D40 and D50 there was no sign of CRALBP immunoreactive MGCs in the ROs (Supplementary Figure 1)

Due to the similarities in protein expression, GFAP expression was not sufficient to assign a specific cell type to the detected signal. But since all ROs were negative for CRALBP, it was likely that the $GFAP^{+}$ cells were astrocytes or other glia that were probably generated from the stem cells as a by-product.

Targeting Notch Signalling to Improve Glia Differentiation Causes a Decrease of Vsx2^{+} Cells

A balance of pro-neural and negative bHLH genes regulated by Notch determines the gliogenic switch. This indicates the necessity of Notch signalling for the transition of late retinal progenitor cells into MGCs. Since there was no evidence of mature MGCs expressing CRALBP in ROs at any timepoint assessed, Notch signalling was targeted in an attempt to enhance MGC differentiation. Therefore, the Notch ligands DLL4 and Jagged1 were added to the cultivation medium of 15-days old ROs. After three days of treatment, ROs were fixed and prepared for cryosectioning followed by immunohistochemical staining.

The treatment from D15 to D18 did not alter the staining pattern of the glia markers NFIa and CRALBP in comparison to untreated ROs on D18. There were no immunoreactive cells for CRALBP or NFIa detectable in treated or untreated ROs (Supplementary Figure 2). Therefore, no positive effect on the differentiation of progenitors into MGCs or an increase of RPCs that develop into late RPCs could be proven. It was concluded that that cell fate decision toward glial cells was not enhanced when Notch signalling was upregulated.

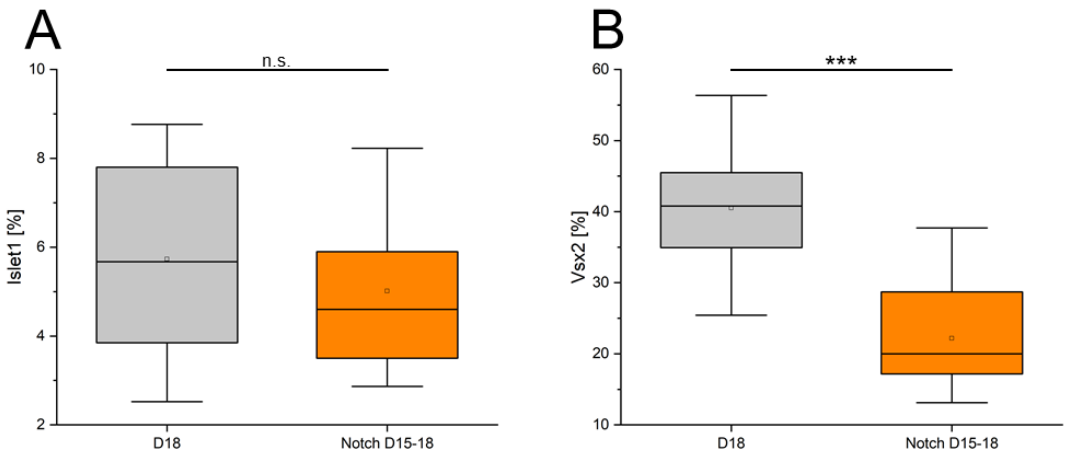


Figure 19: Relative cell number of Islet1 (A) and Vsx2 (B) positive cells located in the neuroepithelium of ROs after three days of treatment with Notch ligands. *Sample sizes A and B: D18 N=3, n=13; Notch D15-18 N=3, n=15. Two sample t-test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.*

Additionally, neuronal markers were stained to see whether neuron composition in the tissue changed independent of glia fate decisions. The measurement of relative cell numbers showed no significant change for cells expressing the ganglion cell marker Islet1 decreasing from 5.73% ± 2.22% to 5.01% ± 1.86% (Figure 19 A). Since this cell type is one of the early generated neurons and its differentiation already peaked before the treatment started, it is possible that the treatment had little to no effect on this specific cell type. The number of cells expressing Vsx2 was 40.52% ± 9.45% in the untreated ROs on D18 and decreased significantly to 22.18% ± 7.14% in the treated ROs (Figure 19 B). Vsx2 is one of the EFTFs and during early development a marker for retinal progenitor cells. The decrease in Vsx2^{+} cells could be the consequence of a shift in the differentiation pattern of early RPCs favouring the differentiation into Vsx2^{-} neurons.

Isolation of Primary Cortical Astrocytes from Mouse Pubs

As a strategy to compensate for the missing MGCs in ROs, primary glial cells were isolated from mice. MGCs and retinal astrocytes share many characteristics even though they have a clear hierarchy determining which tasks are carried out by which cell type. This redundancy can be taken advantage of in co-cultivation setups since it enables the cells to compensate for each other to a certain degree. Therefore, retinal astrocytes were isolated from mouse brains following the protocol of Schildge et al. 2013.

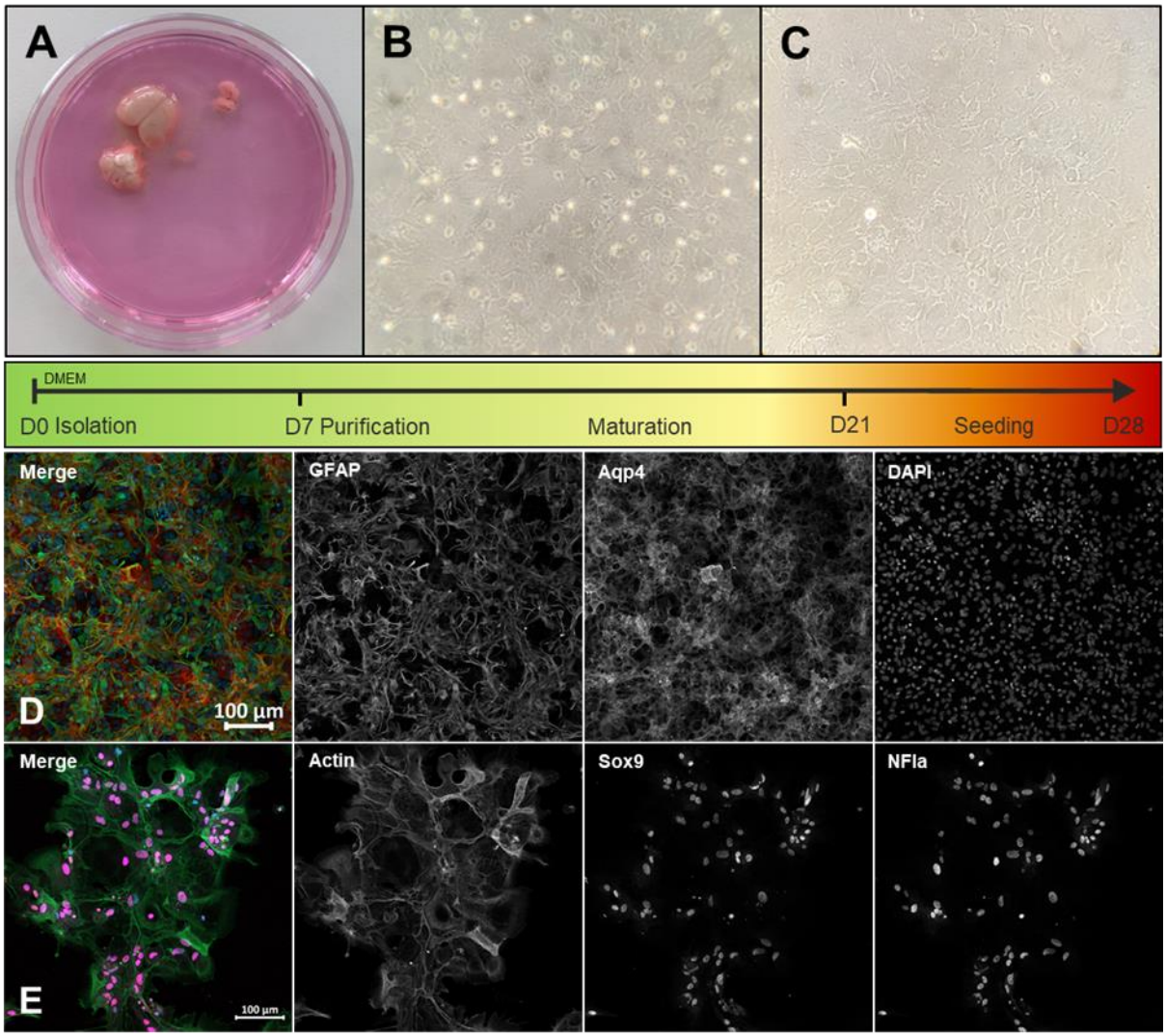


Figure 20: Isolation of primary astrocytes from mouse cortices. *Timeline for the isolation protocol and cultivation with exemplarily pictures showing the dissection of the cortices (**A**) and the astrocyte monolayer before and after the purification step discarding microglia (**B** and **C**). Isolated cells were characterised using the intermediate filament GFAP and the surface marker Aqp4 (panel **D**) as well as the nuclear maker proteins Sox9 and NFIa (panel **E**) to confirm astrocyte identity. The cytoskeleton was stained using Phalloidin and nuclei were stained using DAPI.*

Mouse pubs between P0 and P4 were sacrificed and their brains were isolated. The olfactory bulb and the cerebellum were separated from cortex (Figure 20 A). After removal of the meninges, the cortices were dissociated and a mixed glial cell population was seeded in Poly-D-Lysin coated flasks. The cells adhered to the surface and over the next few days they formed a confluent monolayer. The isolated

astrocytes were flat cells covering the surface of the flask. On top smaller cells could be identified that are rounder in shape and can be morphologically easily distinguished from the astrocytes (Figure 20 B). These cells are a mixed population of oligodendrocyte progenitor cells and microglia that were discarded after the purification process (Schildge et al. 2013).

The isolated astrocytes were cultivated for 21 days to reach the recommended maturity. To confirm astrocyte identity the cells were seeded on glass coverslips and marker proteins were stained. The cells exhibited classical glia markers including the intermediate filament GFAP and the water channel Aqp4 (Figure 20 D). Additionally, the marker proteins Sox9 and NFIa were present in the majority of cells with a nuclear localization (Figure 20 E). This validated the glia identity of the isolated astrocytes and it was therefore continued to design the co-cultivation setup.

Soluble Factor-Mediated Influence of Astrocytes on Whole Retinal Organoids is Insignificant

To substitute the missing MGCs in ROs, primary mouse astrocytes were isolated and used for co-cultivation approaches. For the conjoint cultivation of ROs with primary astrocytes both protocols were aligned to each other (Figure 21 timeline). The astrocytes were preferably seeded after a cultivation period of 21 days to 28 days. Seeding required to be done one day prior to the start of the co-cultivation so that the cells were allowed to adhere and spread. With the start of the co-cultivation the astrocyte medium was discarded and changed to RMM2. ROs were added at D15 as a whole onto the confluent astrocytes. This allowed the glial cells to influence the ROs via soluble factors secreted into the medium with minimal physical contact. The ROs maintained floating and were removed after 3 or 6 days of co-cultivation for fixation, cryosectioning and staining. To determine effects on RO composition, the neuroepithelium was assessed by measuring the relative cell number and distribution of retinal neurons across the apico-basal axis. The investigated markers were used to identify retinal progenitor cells/ later bipolar cells ($Vsx2^+$), ganglion cells ($Islet1^+/Vsx2^-$) photoreceptor progenitors ($Otx2^+$) and mitotic cells ($pH3^+$).

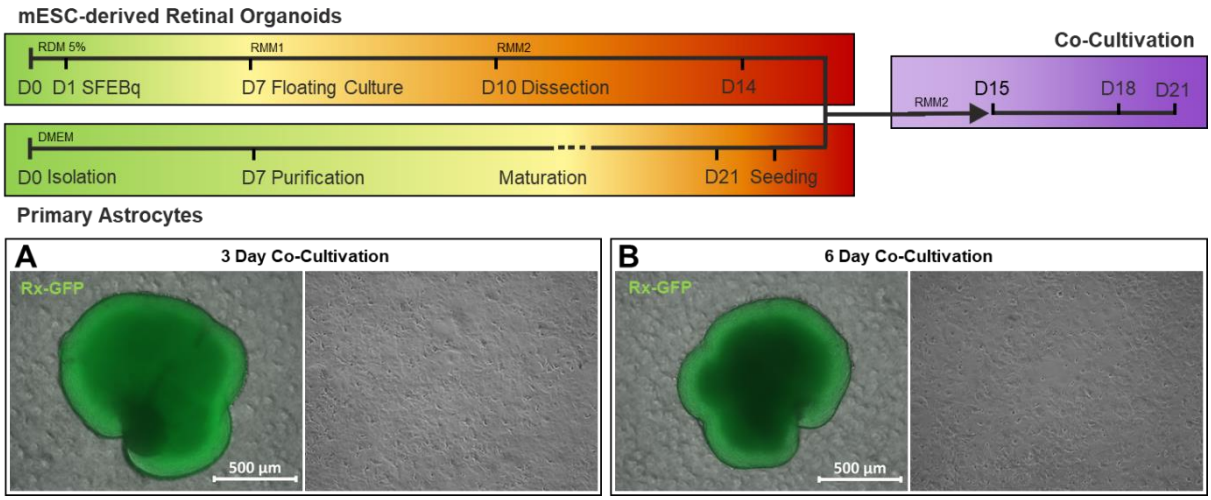


Figure 21: Co-cultivation setup for ROs from mESCs and primary cortical astrocytes from mice. *Timelines for the differentiation of ROs and the isolation of primary astrocytes leading to the conjoint cultivation in a shared system (upper panel). Micrographs of ROs and astrocytes after a co-cultivation period of 3 days (**A**) or 6 days (**B**)*

The ROs were documented before and after each co-cultivation or medium treatment. To assure that possible changes in cell numbers were not the result of the ROs degeneration or that localisation changes were not the result of loss of structure of the epithelium. All ROs maintained bright Rx-GFP signal during the treatment and were morphologically similar to the untreated ones (Figure 21 A and B). It was the main goal to keep the culture conditions in favour of the ROs and was therefore decided to perform the co-cultivation in the retinal maturation medium RMM2 normally used for the cultivation of ROs to avoid changes of the medium composition by switching to astrocyte maintenance medium (Astro MM). Because of this, the ROs were comparable to the untreated control cultivated in RMM2 and possible effects could be pinpointed down to the co-cultivation procedure.

The co-cultivation (CO) of ROs with cortical astrocytes from D15 to D18 did not lead to significant changes in the composition of the neuroepithelium of ROs. There were no changes in the relative numbers of Islet1$^+$, Vsx2$^+$ or Otx2$^+$ cells even after doubling the duration of the co-cultivation from three to six days (Figure 22 and 23). The astrocytes did not die, detach or change their morphology in RMM2 (Figure 21 A and B) but the possibility could not be excluded that they still experience stress and secrete fewer or a different combination of soluble factors into the medium. To address this, CM was collected from astrocytes in Astro MM. If the astrocytes produce soluble factors of any kind influencing the growth or differentiation of ROs the CM should be enriched in these factors. It was added to the ROs as supplement to the culture medium. As a result, three of the four proteins monitored showed a significant change in relative cell numbers after three days (Figure 22 and 23).

At D18 in ROs, 5.73% ± 2.22% of cells were positive for Islet1. Treatment with CM increased the cell numbers of Islet1$^+$ cells to 8.77% ± 2.44 % after three days and to 6.29% ± 1.41% after six days. Without CM treatment only 3.66% ± 1.15% of cells remain immunoreactive for Islet1 on D21. The total amount of Islet1$^+$ cells continued to decrease over time (Figure 22 A) but the treatment with CM was able to decelerate the process (Figure 23 A and B). After six days of cultivation with CM supplemented to the medium the ROs included more Islet1$^+$ cells than the untreated ROs at D18. Three-day treatment also led to the decrease of Vsx2$^+$ cells to 31.29% ± 7.22% compared to the control ROs on D18 making up 40.52% ± 9.45% of the neuroepithelium. While the numbers naturally decreased to 29.88% ± 10.75% on D21 the cultivation with CM from D15 to D21 further decreased the portion of Vsx2$^+$ cells to 20.18% ± 5.94%. The treatment between D15 and D18 therefore caused a loss of cells that made up approximately 9% while the following three days account for additional 10%. This indicates that the loss is an ongoing process and not limited to a cell fate decision on one specific time point during the experiment.

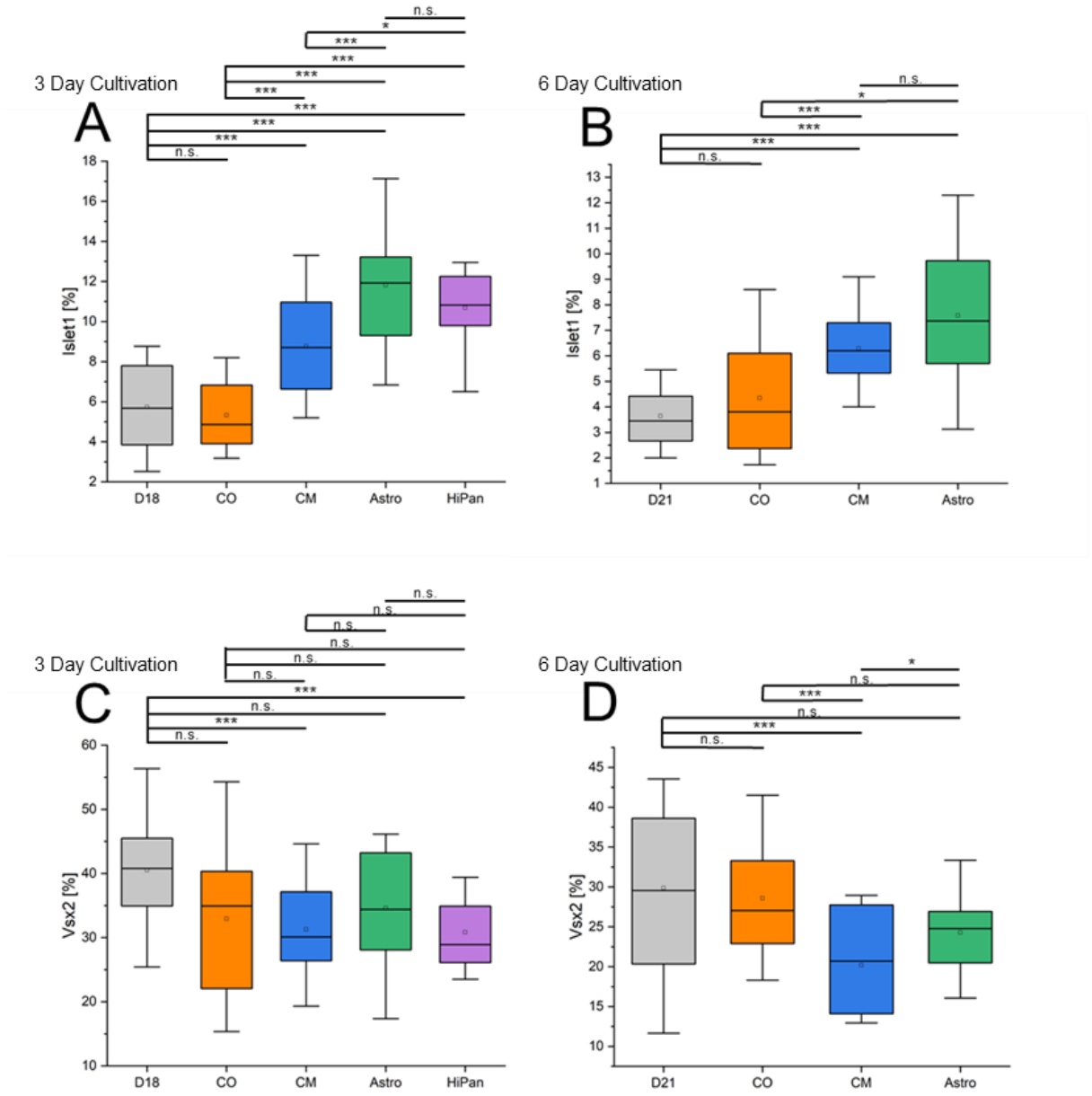


Figure 22: Interneuron quantification in ROs after three or six days of co-cultivation or treatment with different media compositions compared to untreated ROs on D18 or D21. *Relative cell numbers of Islet1⁺ (**A** and **B**) or Vsx2⁺ cells (**C** and **D**) measured after co-cultivation with astrocytes (CO), treatment with conditioned medium (CM), cultivation in astrocyte medium (Astro) or RMM2 with heat inactivated serum (HiPan). Sample sizes A and C: D18 N=3, n= 13; CO: N=3, n=15; CM: N=3, n=15; Astro: N=3, n=15; HiPan: N=3, n=14; B and D: D21: N=3, n=15; CO: N=3, n=15; CM: N=3, n=15; Astro: N=3, n=15. Two sample t-test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.*

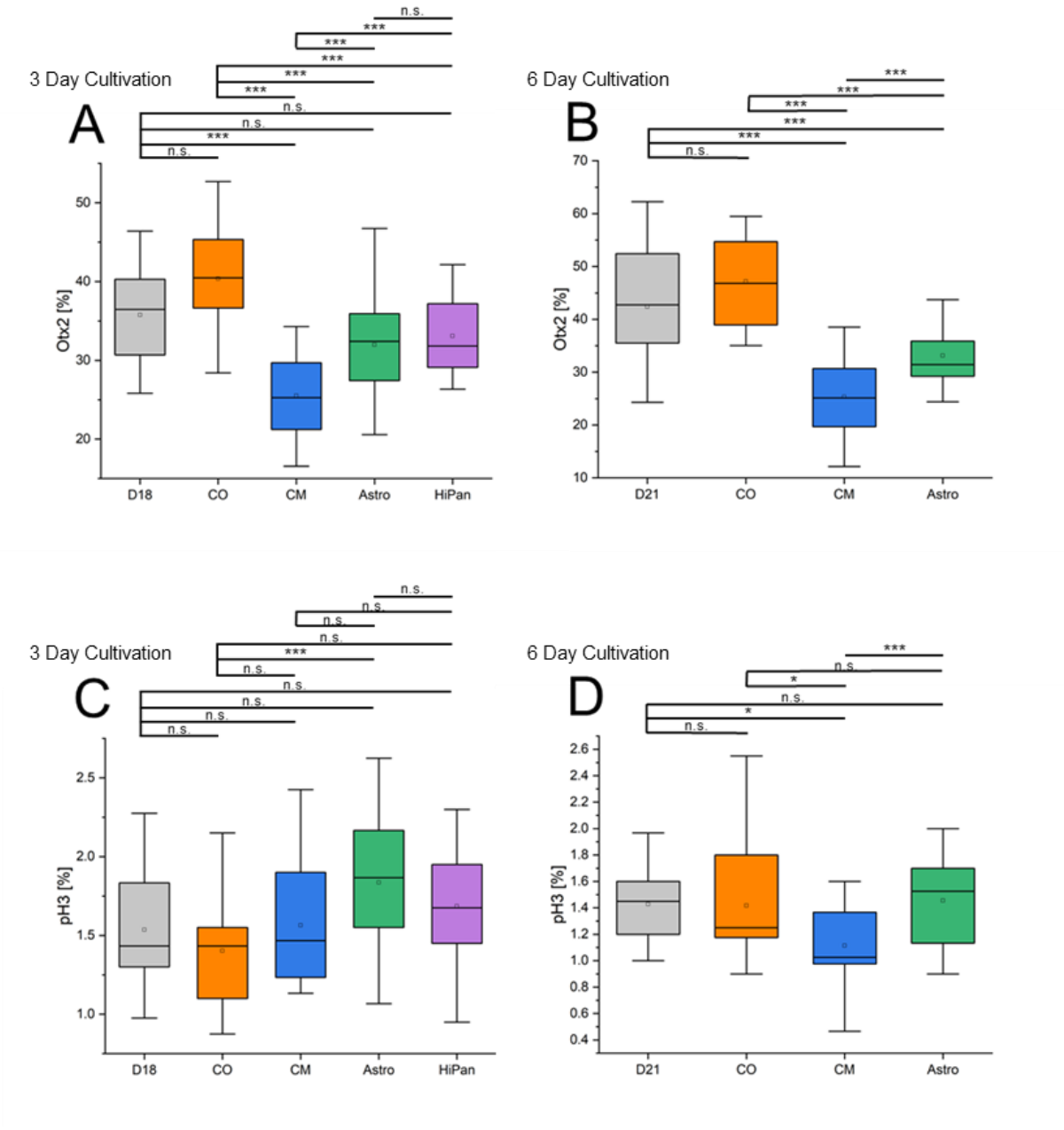


Figure 23: Quantification of photoreceptors and mitotic cells in ROs after three or six days of co-cultivation or treatment with differing media compositions compared to untreated ROs on D18 or D21. *Relative cell numbers of Otx2⁺ (**A** and **B**) or pH3⁺ cells (**C** and **D**) measured after co-cultivation with astrocytes (CO), treatment with CM, cultivation in astrocyte medium (Astro) or RMM2 with heat inactivated serum (HiPan). Sample sizes A and C: D18 N=3, n= 14; CO: N=3, n=15; CM: N=3, n=15; Astro: N=3, n=15; HiPan: N=3, n=14; B and D: D21: N=3, n=15; CO: N=3, n=15; CM: N=3, n=15; Astro: N=3, n=15. Two sample t-test: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.*

With 35.76% ± 6.48% photoreceptors made up a big portion of the retinal epithelium on D18. Further increasing to 42.39% ± 10.63% at D21 under regular culture conditions (Figure 23 A). Treatment with CM causes a drastic decrease in Otx2⁺ cells reducing its portion to 25.51% ± 5.26% after three days and 25.31% ± 7.58% after six days of cultivation (Figure 23 A and B). The treatment with CM showed a big impact in the time period between D15 and D18 but stagnated afterwards showing no further decrease after elongated treatment until D21. The cells seem to be more prone to the treatment during the first three days but remain unaltered therafter.

However, the cell numbers generated by co-cultivation and the treatment with CM diverged, even though the general mechanism targeted was the same. In both cases soluble factors mediated signalling was enabled to alter cell composition. But since the CM was collected from the astrocytes in Astro MM, it was necessary to determine whether the medium composition caused the changes.

For the Islet1$^+$ cells, relative cell numbers increased to 11.82% ± 3.25% after a three-day cultivation period in Astro MM demonstrating a continuous increase in cell numbers with increased portions of Astro MM in the culture medium. After six days the cultivation in Astro MM was able to recreate the effect of the CM treatment causing a significant increase in comparison to the untreated control on D21 while being insignificantly different from the CM group (7.58% ± 2.42%) (Figure 22 A and B). The cultivation of ROs in Astro MM from D15-D18 led to a relative cell number of 34.61% ± 8.70% for Vsx2$^+$ cells in the epithelium. This value lies below the control and co-cultivated samples but above the CM samples. But since the differences were small, the Astro MM condition did not show a significant difference. This changed with longer cultivation time. After six days, the Astro MM treated ROs contained 24.27% ± 4.82% Vsx2$^+$ cells and were significantly decreased to both, the control and the co-cultivated ROs but increased compared to the CM. The same tendency was observed analysing the Otx2$^+$ cells. After the treatment with Astro MM from D15 to D18 the relative cell number was 31.98% ± 6.68% which lays below the number of Otx2$^+$ cells in the control and the co-cultivated ROs but above the number in ROs after the treatment with CM. After three days of treatment the cell number is already significantly decreased compared to the co-cultivated ROs and increased compared to the CM treated ROs (Figure 23 A). This positioning was similar after six days of cultivation in Astro MM where Otx2$^+$ cells made up 33.14% ± 5.34% of the epithelium and now decreased significantly compared to the control as well (Figure 23 B). In parallel, the number of mitotic cells was analysed in the conducted experiments by staining of pH3. Immunoreactive cells in ROs at D18 made up 1.54% ± 0.38% of the total cells in the epithelium and remained constant for ROs at D21 (Figure 23 C). For the three-day experimental procedures this was not altered by any of the conditions tested. After the prolonged treatment of six days the cultivation with CM decreased the number of pH3$^+$ cells to 1.11% ± 0.30%. The co-cultivation or the treatment with Astro MM showed no effect even after elongation of the cultivation period (Figure 23 D).

Interestingly, the number of cells positive for each protein of interest measured after three days could be recreated by the cultivation of the ROs in RMM2 with heat inactivated serum (Figure 22 and 23). Thereafter, immunoreactive cells made up 30.82% ± 5.41% for Islet1, 30.82% ± 5.41% for Vsx2, 33.09% ± 5.35% for Otx2 and 1.69% ± 0.41% for pH3 showing no significant difference to the Astro MM treated samples in all cases. This indicated that the denaturation of serum proteins is most likely one of the main drivers responsible for the changes in epithelium composition.

Additionally, to the quantificational analysis of the neuroepithelium, it was investigated whether the apico-basal polarity in the ROs was altered. Influences on the localisation of cell types in the epithelium can occur independently of the relative cell numbers and were therefore analysed separately. A shift in epithelial localisation could indicate, for example, a different state of maturation, influences on cell migration or organisation of the tissue.

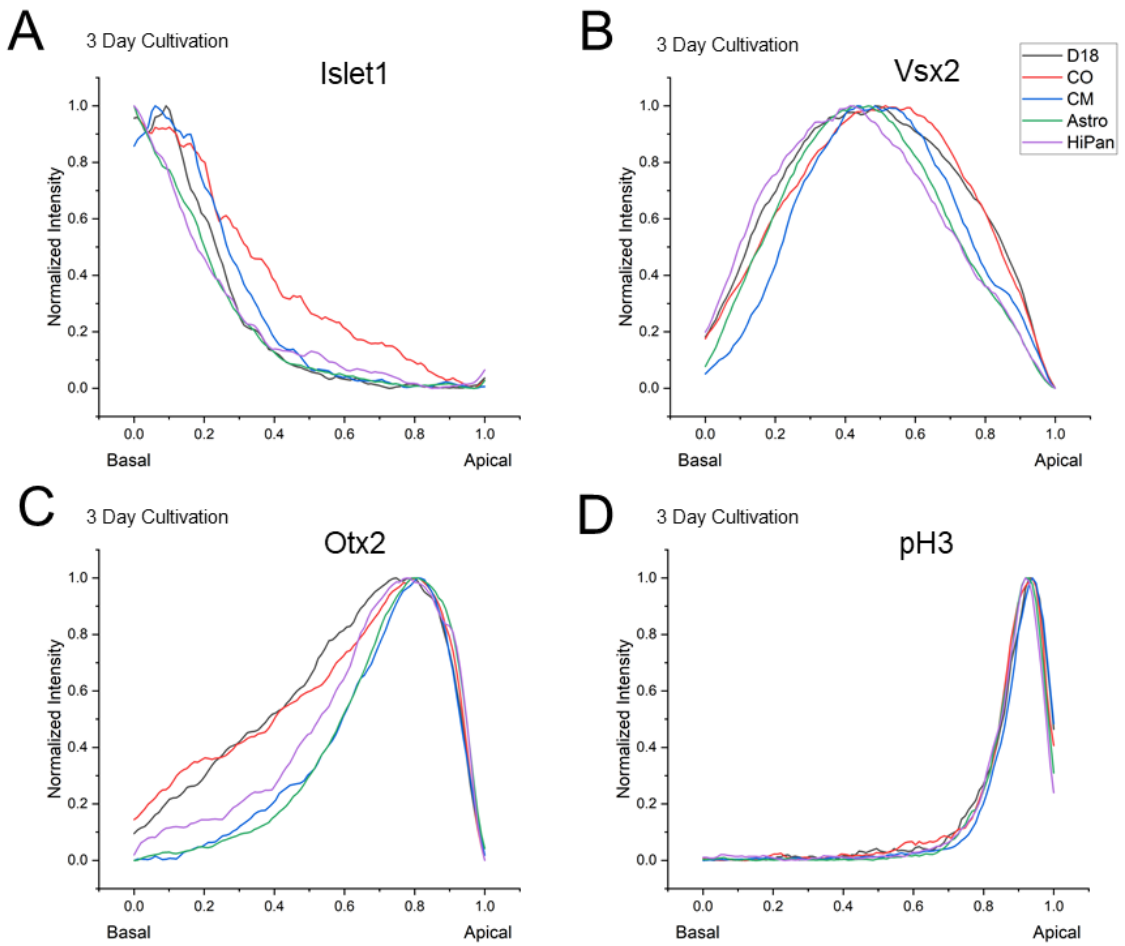


Figure 24: Localisation of neurons and mitotic cells after 3 days of co-cultivation with cortical astrocytes or treatment with different medium compositions. *Line measurements show the apico-basal distribution tendencies of Islet1⁺(**A**), Vsx2⁺(**B**) and Otx2⁺(**C**) neurons and pH3⁺ mitotic cells (**D**) in the neuroepithelium of ROs at D18. Cellular localisation was compared to ROs after three days of co-cultivation with cortical astrocytes (CO) treatment with Astrocyte CM (CM), unconditioned Astro MM (Astro) or treatment with RMM2 with heat inactivated serum (HiPan).*

The plots resulting from the line measurements show distinct patterns with minor fluctuations for each marker. The conditions tested did not influence the general organisation of the neuroepithelium after three days of treatment (Figure 24). Staining for Islet1 expressing cells exhibited a clear preference for the basal side. This can be seen in the plot by a sharp maximum on the basal side and a decrease of the curve in apical direction where only a small portion of cells was detected in the epithelium (Figure 24 A). Even though the co-cultivated ROs showed a slightly flattened decrease of the curve compared to all other samples the preference for basal localisation remained. The cells positive for Vsx2 could be mainly found in the central part of the epithelium (Figure 24 B). The curves of all conditions were in close proximity and comparable sharp with no sample standing out after the treatment. Interestingly, this was not the case for the Otx2⁺ cells. Although the maxima were all at the apical side, the course of the curves differed slightly. The co-cultivated ROs showed close similarity to the untreated control organoid at D18. Meanwhile, treatment with the CM, Astro MM or RMM2 with heat inactivated serum for three days resulted in a downwards shift of their curves in the central to basal region. This could indicate a higher specificity of the Otx2⁺ cells to the apical side in these conditions resulting in fewer Otx2⁺ cells detected in the basal region (Figure 24 C). Mitotic cells positive for pH3 were strictly confined to the apical side and remained unaltered regardless of the treatment (Figure 24 D).

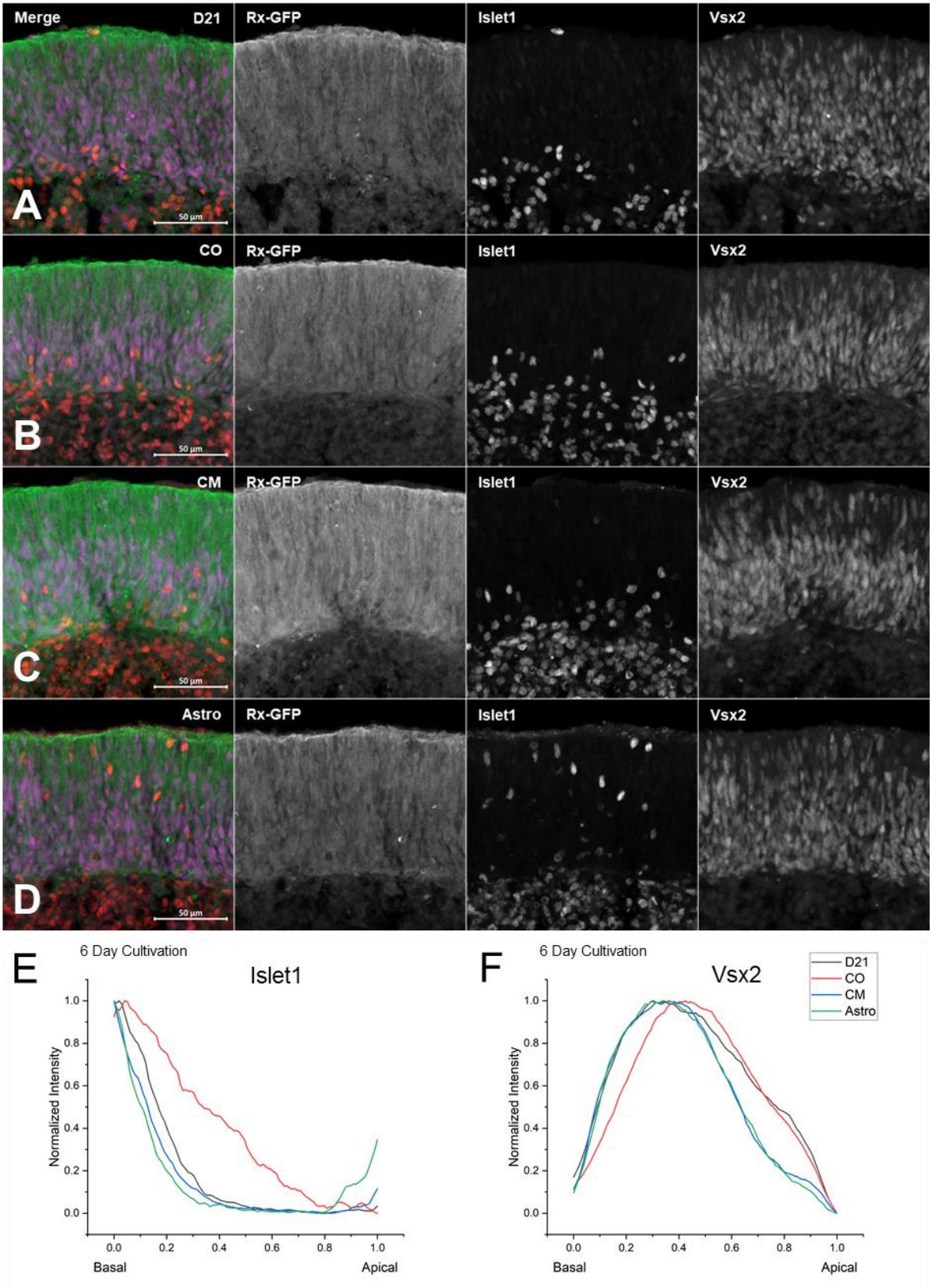


Figure 25: Epithelial distribution of interneurons after six days of co-cultivation with cortical astrocytes or treatment with different medium compositions. *Micrographs show the localisation of immunoreactive cells for Islet1 and Vsx2 in the epithelium of ROs after six days of co-cultivation with cortical astrocytes (**B**, CO), cultivation with their CM (**C**) or astrocyte MM (**D**, Astro) in comparison to the untreated control (**A**, D21). Graphical depiction of the measured localisations of Islet1⁺ (**E**) and Vsx2⁺ cells (**F**).*

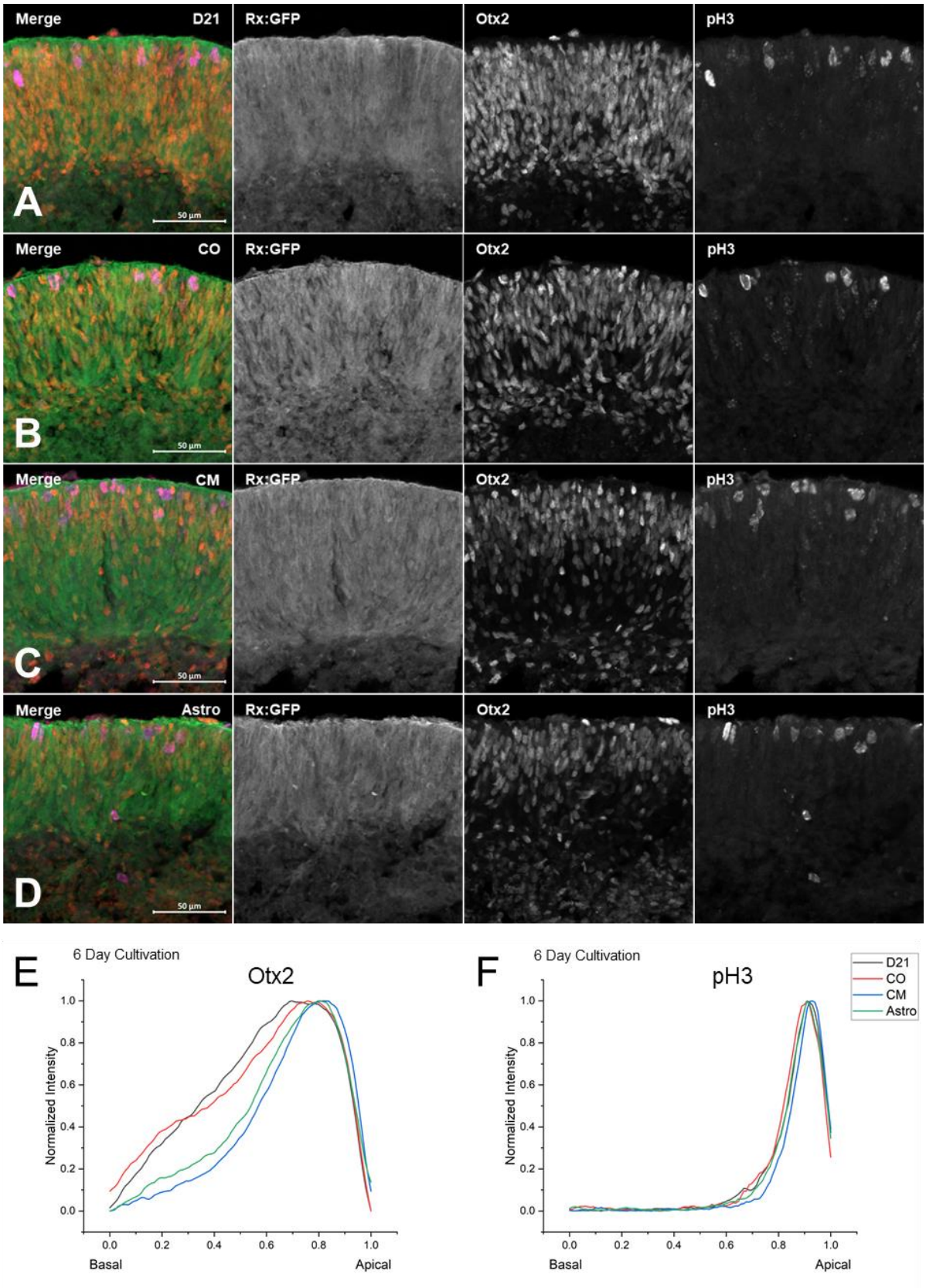


Figure 26: Epithelial distribution of photoreceptors and mitotic cells after six days of co-cultivation with cortical astrocytes or treatment with different medium compositions. *Micrographs show the localisation of immunoreactive cells for Otx2 and pH3 in the epithelium of ROs after six days of co-cultivation with cortical astrocytes (**B**, CO), cultivation with their CM (**C**) or unconditioned astrocyte MM (**D**, Astro) in comparison to the untreated control (**A**, D21). Graphical depiction of the measured localisations of Otx2⁺ (**E**) and pH3⁺ cells (**F**).*

Micrographs of the ROs at D21 give a good impression of the general localisation of the cells of interest. With longer cultivation periods, differences in the apico-basal distribution of the retinal neurons start to appear. These are reflected in the line measurements. Islet1$^+$ cells show their characteristic restriction to the apical side for all conditions (Figure 25). After the co-cultivation with astrocytes for six days the same effect, already seen after three days of co-cultivation, was even stronger. The cells showed increased localisation on the basal side but the curve was flattened and reached further into the central region of the epithelium. In these ROs Islet1$^+$ cells did not form a border as sharp as in the other conditions that were comparable to each other. The curves of the control organoid, the CM and the Astro condition shifted to the left seemingly increasing the basal specificity of the Islet1$^+$ cells over time. Notably, in ROs that were treated with Astro MM the curve additionally increased on the apical side. It is noticeable in the micrographs that there are some Islet1$^+$ cells located inside the epithelium (Figure 25 D). The Vsx2$^+$ cells were firstly expressed throughout the whole neuroepithelium and over time get located more basally. At D21, a slight shift in basal direction can be observed in the control organoids compared to earlier timepoints (Figure 15 D). The co-cultivated ROs as well as the ROs treated with CM or Astro MM for six days were even further shifted to the left following this tendency. The graphs also show that Vsx2$^+$ cells in regular ROs and the co-cultivated ROs spread further in the apical direction compared to the Astro MM and the CM treated ROs (Figure 25 F). The Otx2$^+$ cells followed the same distribution as after the three-day treatment. Most cells were located at the apical side which represents the *in vivo* localisation of Otx2$^+$ photoreceptors (Figure 26). The similarity between the ROs at D21 and the co-cultivated ROs was maintained even after longer cultivation (Figure 26 E) The distribution can also be observed in the micrographs. In the ROs and the co-cultivated ROs, Otx2$^+$ cells spread further into the basal region of the neuroepithelium (Figure 26 A and B). The Otx2$^+$ cells in ROs treated with CM or the astrocyte MM exhibited a stricter limitation to the apical side with only a few cells expressing Otx2 in the central to basal region of the epithelium. (Figure 26 C and D) This indicated a similarity of this samples that was also seen in the resemblance of their curves (Figure 26 E). Mitotic cells positive for pH3 did not show any alteration in their localisation profile regardless of the treatment. They remain strictly limited to the apical side and this was consistent in the micrographs as well as in the line measurements (Figure 26).

The co-cultivation of ROs with cortical astrocytes did not result in any quantitative or organisational differences that could validate an improvement of the current culture conditions. Additionally, the cultivation of ROs in Astro MM (Astro) and in RMM2 with heat inactivated serum (HiPan) leads to the conclusion that the significant changes the CM caused were not attributable to secreted factors from the primary astrocytes but were due to differences in medium composition, especially the heat inactivation of serum components. Therefore, the focus was shifted towards a cultivation system integrating the astrocytes in a 3D environment were cell-cell contact mediated signalling was possible.

Dissociated Retinal Organoids can be Reaggregated and Recreate Organoid Organisation

One of the great characteristics of retinogenesis is the autonomous differentiation and self-organisation of retinal neurons from a common progenitor pool to build complex neuronal circuits. Thus, first it was examined whether mixed retinal cells were still able to reorganise after complete dissociation into single cells. Previous work done in our group showed some degree of reorganisation of dissociated retinal neurons when seeded into 3D printed scaffolds (S. Keppler 2021). In this work the approach is characterised by seeding of the cells into low adhesion well plates to generate free-

floating three-dimensional aggregates that could easily be sectioned for further analysis. The ROs were cultured until D14 where the maturation process begins and ROs are remaining in RMM2 without further supplement changes. The seeding of organoid-derived retinal cells resulted in the formation of aggregates further referred to as reaggregated ROs. The cells formed an aggregate one day after seeding that was disorganised but contained primarily Rx-GFP$^+$ cells. Thereafter, the reaggregated ROs rapidly grow and formed first signs of an epithelium until day four after seeding. After four days they were transferred into a petri dish and were cultivated for additional 10 days before being fixed. After one week they displayed similar morphology to the regular cultured ROs (Supplementary Figure 5).

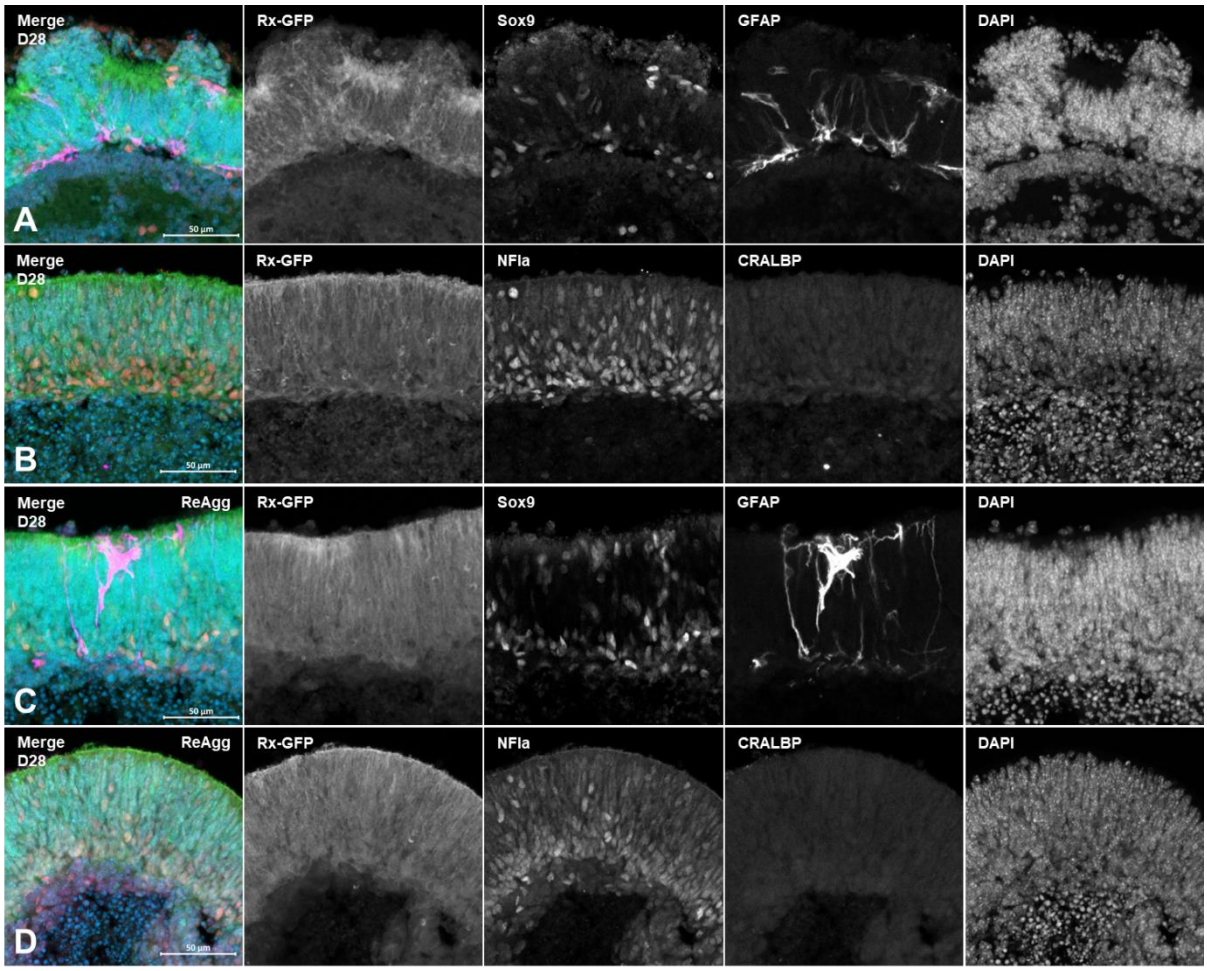


Figure 27: Cross-section through the retinal epithelium of ROs in comparison to reaggregated ROs after a total cultivation period of 28 days*. Immunostainings show the localisation of cells expressing the late retinal progenitor markers Sox9 and NFIa, the astrocyte marker GFAP and the MGC marker CRALBP in regularly cultivated ROs (**A** and **B**) compared to reaggregated ROs (**C** and **D**).*

After a total cultivation period of 28 days, immunochemical staining revealed that the reaggregated ROs are not only morphologically similar to the regularly grown ROs. They formed an Rx-GFP$^+$ neuroepithelium that established an apico-basal polarity (Figure 27, Rx-GFP and DAPI channel). All cell types detected in the ROs were present after reaggregation, and their organisation in the tissue was re-established accordingly to the *in vivo* localisation. This included the positioning of late retinal progenitor cells on the basal side (one example, Figure 27, NFIa channel), ganglion cells on the basal side, as well as photoreceptors and mitotic cells on the apical side (Figure 28 and 29). Reaggregated

ROs, like the regular ROs, rarely contained GFAP$^+$ glial cells and were always negative for the MGC specific marker CRALBP (Figure 27). Seemingly, the reorganisation had no effect on the differentiation of glia cell types in the epithelium. Therefore, the planned integration of glia cells into the microenvironment could be controlled by the number of glia specific markers.

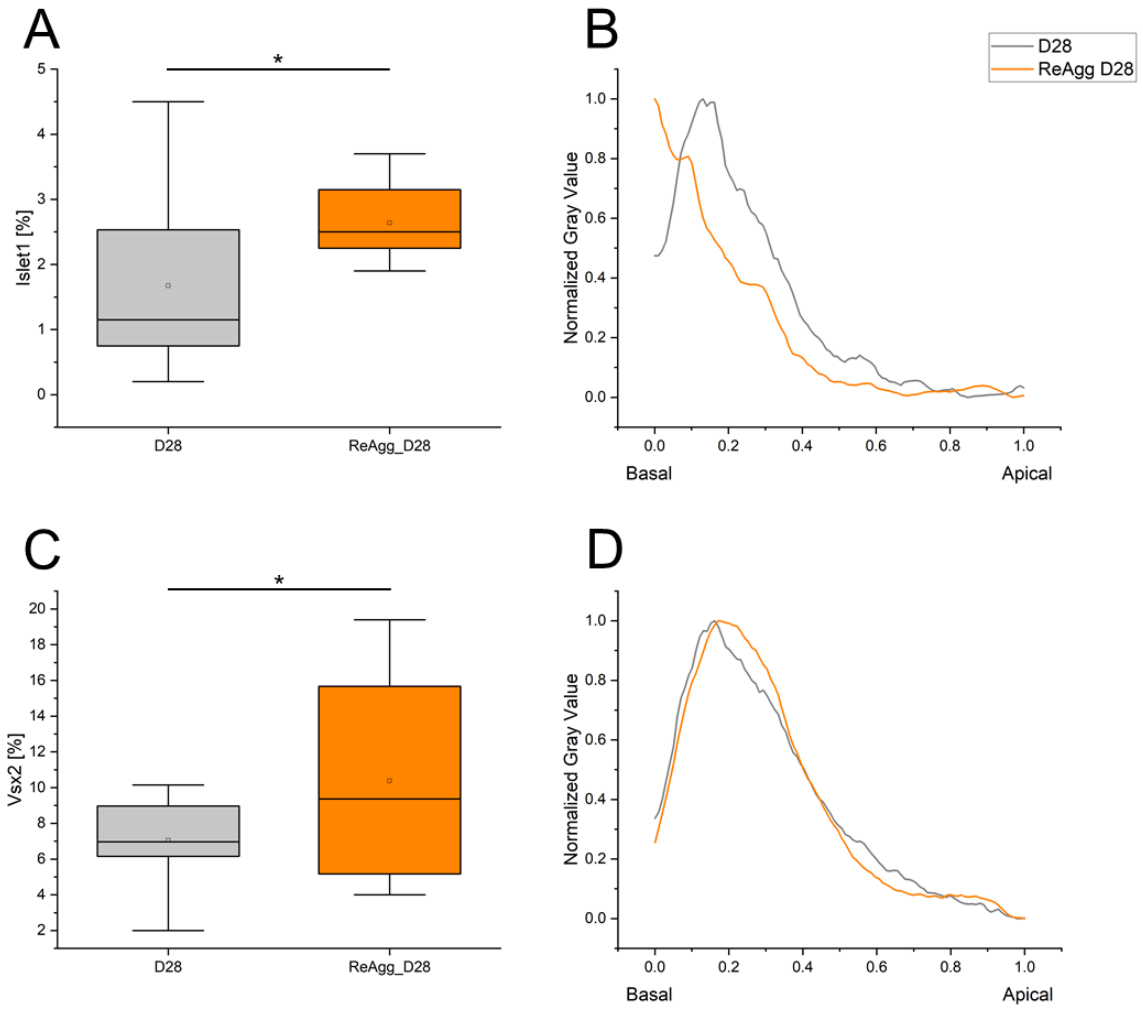


Figure 28: Profile of Islet1$^+$and Vsx2$^+$ cells in ROs and reaggregated ROs after a total cultivation of 28 days. *Relative cell numbers of Islet1$^+$ (**A**) and Vsx2$^+$ (**C**) cells in reaggregated organoids compared to regular ROs and their distribution across the epithelium (**B** and **D**). Sample sizes: D28 N = 3, n = 15, ReAgg_28: N = 3, n = 15. Two sample t-test: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.*

Further characterisation showed that the reaggregated ROs and the regular ROs at D28 are not only morphologically alike. They also show that the same general organisation and the apico-basal distribution of all analysed cell types was similar (Figure 28 and 29). The quantification of cell numbers, however, did show some differences. The relative cell number of Iset1$^+$ cells was significantly increased in reaggregated ROs making up a portion of 2.64% ± 0.52% compared to 1.67% ± 1.30% in regularly cultivated ROs on D28. The same was seen for the Vsx2$^+$ cells. Their portion of epithelial cells in reaggregated ROs was 10.37% ± 5.24% which was significantly more than in ROs (7.05% ± 2.15%) (Figure 28 C). This means that the reaggregation process positively affected the cellular composition of the ROs and could be beneficial for some cell types. Notably, the Vsx2$^+$ cells showed the same time

depended shift to the basal region. This gives the impression that the cells go through the same organisational process in parallel and this time dependent development was not disrupted by the dissociation on D14.This was also noticeable for the Otx2$^+$ cells. At earlier timepoints, like D18 or D21, the cells were accumulated at the apical side (Figure 29 B). They lose this organisation over time and are localise more in the central part of the epithelium at D28. The reaggregation on D14 did neither affected the localization pattern over time nor did it alter the relative number of Otx2$^+$ cells (D28: 43.11% ± 7.57% and ReAgg: 43.09% ± 7.09%) (Figure 29 A and B). Surprisingly, the number of mitotic cells increased from 0.46% ± 0.18% to 1.22% ± 0.42% in the reaggregated ROs (Figure 29 C). It was not clear what caused the differences in mitotic activity of the cells in comparison to the regular ROs. The portion is closer to the numbers measured in younger ROs, which contain more RPCs that undergo division before differentiation (D21: 1.43% ± 0.28% see Figure 29 C). Maybe more progenitor cells proliferated in the reaggregated ROs, while in the regular ROs at D28 cells are terminally differentiated and the mitotic activity is lost.

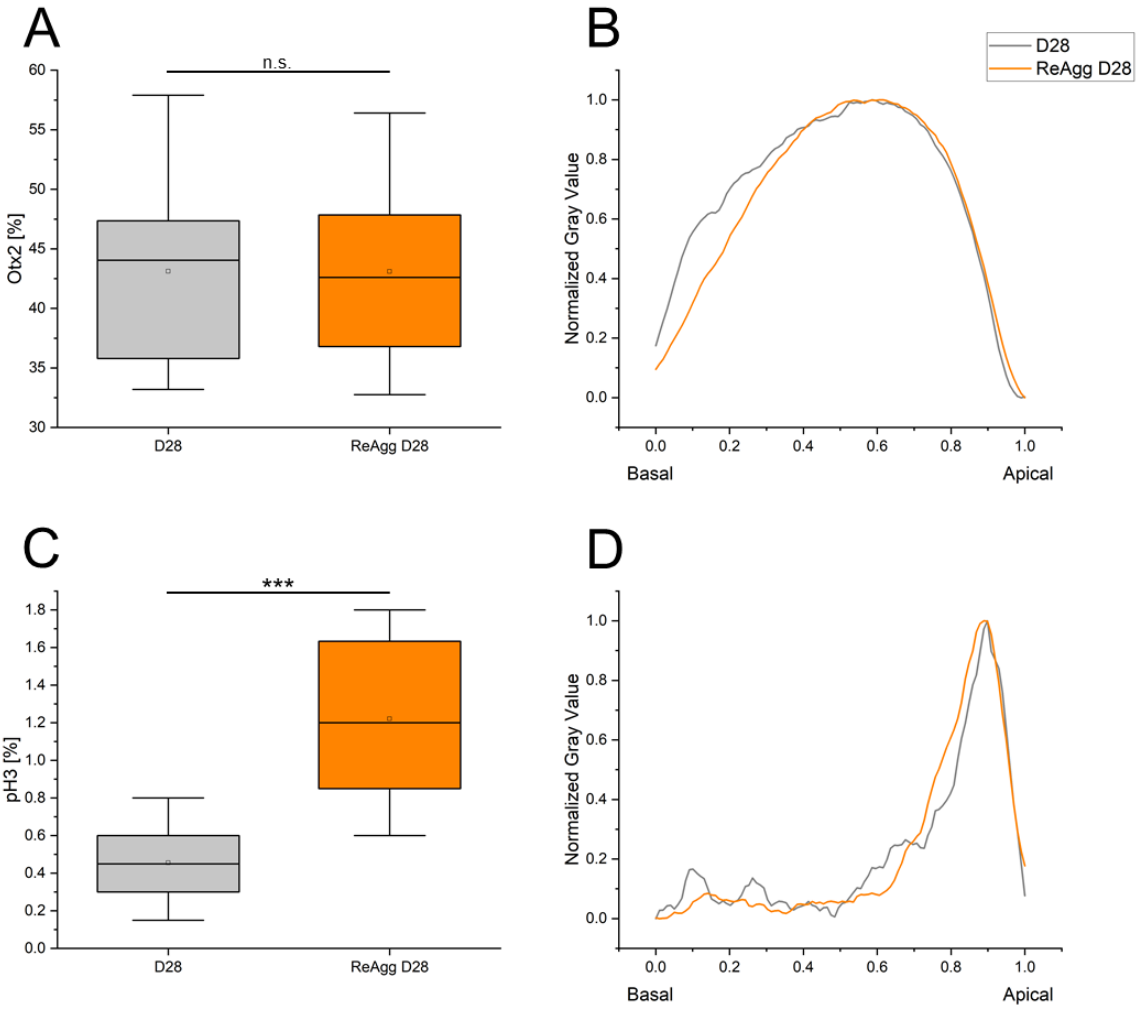


Figure 29: Profile of Otx2$^+$and pH3$^+$ cells in in ROs and reaggregated ROs after a total cultivation of 28 days. *Relative cell numbers of Otx2$^+$(**A**) and pH3$^+$(**C**) cells in reaggregated organoids compared to regular ROs and their distribution across the epithelium (**B** and **D**). Sample sizes: D28 N = 3, n = 15, ReAgg_28: N = 3, n = 15. Two sample t-test: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.*

Integration of Astrocytes in Reaggregated Organoids Produced Disorganised Assembloids

Glial cells in the retina regulate water and ion homeostasis and form cellular connections to various neurons across all retinal layers to maintain neuronal function. To potentially recreate such a neuroprotective environment inside the ROs, it requires a system for conjoint cultivation called assembloids. After the characterisation of reaggregated ROs, the first assembloids were generated in a non-guided manner for the retinal cells. This means that retinal cells and astrocytes were mixed as single cells and subsequently seeded jointly to form assembloids.

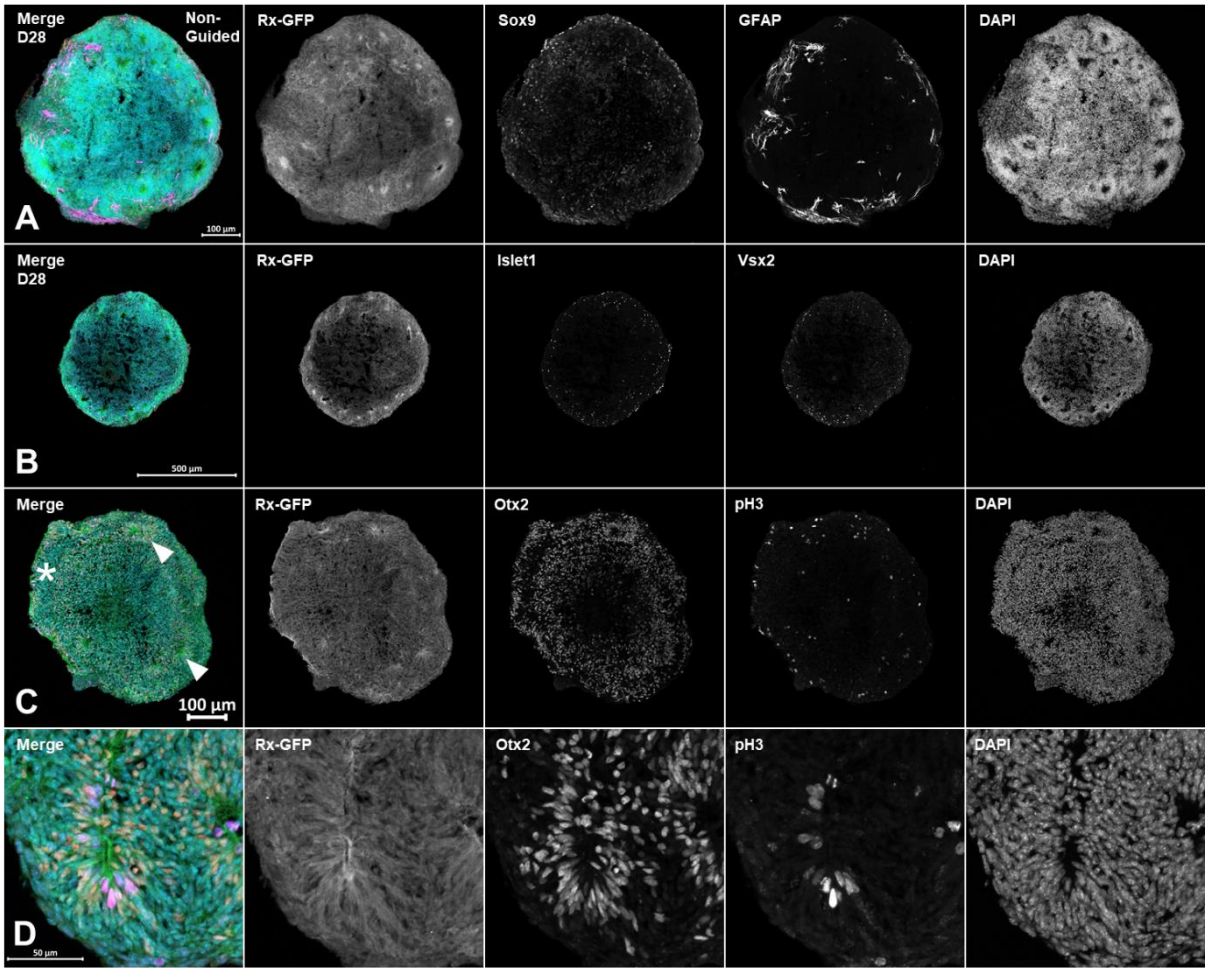


Figure 30: Assembloids generated from organoid-derived retinal cells and cortical astrocytes in an unguided manner. *Cross-sections display the portions of retinal neurons (Rx-GFP⁺) and incorporated astrocytes (GFAP⁺) after a total cultivation of 28 days (**A**). Presence of retinal neurons positive for Sox9, Islet1, Vsx2, Otx2 revealed disorganisation of the tissue (**B** and **C**). Exceptions were rarely occurring epithelial like regions (asterisk) and structured neuronal rosettes (**C** arrowheads and **D**).*

The integration of astrocytes in the assembloid was verified by staining of GFAP and could show that after 14 days of cultivation, numerous GFAP⁺ cells were present (Figure 30 A). Nevertheless, immunohistochemical staining could not clarify whether the cells were newly generated in the assembloid or remained from the initial addition of astrocytes on D14. But since all assembloids analysed contained high numbers of GFAP⁺ cells compared to the regular ROs or the reaggregated ROs

at D28, it was most likely that the astrocytes were successfully integrated on D14 and survived the cultivation until D28. As a consequence of the integration of astrocytes, retinal neurons showed a disorganised arrangement and were not able to reconstruct a continuous epithelial layer (Figure 30). Islet1^{+} and Vsx2^{+} cells were present in the assembloids but were dispersed in the outer regions of the assembloid with no clear organisation (Figure 30 B). Polarity was limited to very rarely occurring epithelial sections (Figure 30 C asterisk) and frequent neuronal rosettes (Figure 30 D). In these microenvironments, the apico-basal organisation was at least partially maintained. In the central region of the rosettes, for example, the pH3^{+} and Otx2^{+} cells co-localised, suggesting an apical identity of these regions. This means the cells did not lose the ability to self-sort and form polarised structures, but on a larger scale, this process was altered by the introduction of the astrocytes into the system. The integration could interfere with cell-cell contact-mediated sorting mechanisms, averting the formation of one polarised epithelium.

Generation of Structured Assembloids did not Rescue Neuroepithelium Formation

In an attempt to compensate for the astrocyte-mediated structural loss, a more guided approach was designed. To help dissociated retinal cells regain their apico-basal orientation, an artificial basal side was introduced. This mimics the *in vivo* situation where retinal astrocytes reside in the most basal layer of the retina. To achieve this, an aggregate solely containing astrocytes was generated one day in advance, which was subsequently enveloped by the seeded retinal neurons and therefore integrated in the central region of the assembloid. Staining of the intermediate filament GFAP confirmed the incorporation of the glia core in the central part of the assembloid (Figure 31 A). Due to the structural differences of the cells in the glia core, they were clearly identifiable in all cross-sections even when no glia specific marker was stained (Figure 31 asterisk). The integration of a glia aggregate in the assembloid was verified in 12 out of 13 assembloids assessed and therefore had high reproducibility. Just as in the unguided approach, the assembloids contained the neuronal cell types positive for Islet1, Vsx2 and Otx2 and some $pH3^+$ mitotic cells. It was noticeable that many $Islet1^+$ cells were also immunoreactive for Vsx2, indicating later born bipolar cells, regardless of the epithelial structure (Figure 31 D arrowheads).

But even though the astrocytes were integrated to provide a defined basal side, their presence still disrupted the collective organisation of the neurons, and no epithelium was formed (Figure 31). The retinal cells were seemingly not able to identify the introduced basal side and orient themselves accordingly. Additionally, the neurons enclosing the glia ore were not able to self-sort, even though they only experience cell-cell contact from other neurons in this region without the interference of astrocytes. Contact-mediated sorting seemed to be enough to guide the reorganisation in the reaggregated ROs but was not sufficient to reorganise the outer parts of the assembloids. It was therefore concluded that the cortical astrocytes are probably not suited to complement the reaggregated ROs in a way that is not at the expanse of basic organisation.

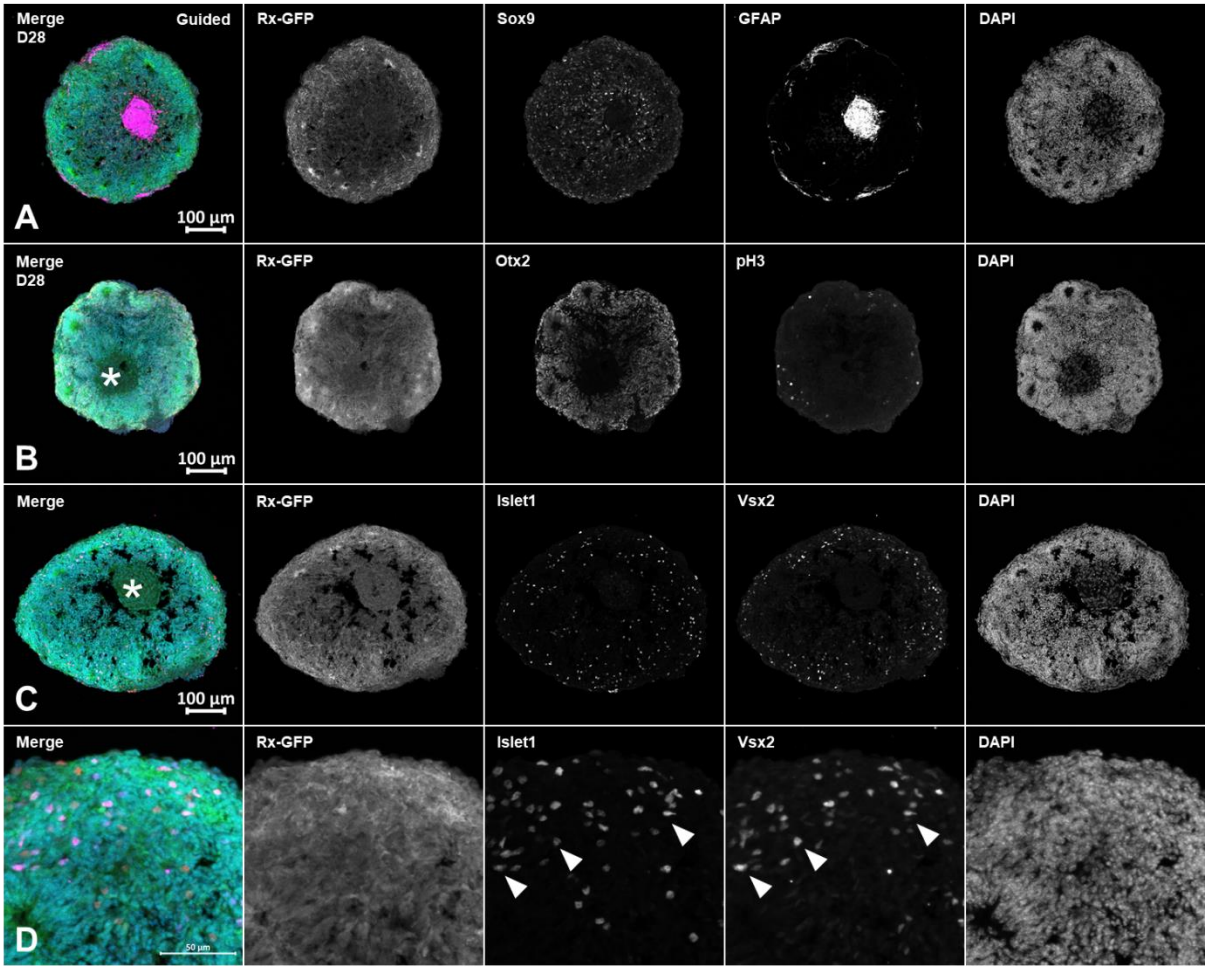


***Figure 31**: **Assembloids generated from organoid-derived retinal cells and aggregated cortical astrocytes in a guided manner.** Micrographs demonstrate the incorporation of the GFAP⁺ glia core inside the central region of the assembloids that can also be identified by the density of the nuclei (asterisk) (**A**, **B** and **C**). Rx-GFP⁺ retinal cells encased the astrocytes but failed to reorganise a continuous epithelium. They formed neuronal rosettes containing cells positive for Sox9, Islet1, Vsx2 and Otx2. Islet1⁺ cells mainly represent bipolar cells, as demonstrated in the co-staining for Vsx2 (**C** arrowheads and **D**).*

Discussion

Characterisation of Retinal Organoids

For the generation of ROs, mESCs were seeded into low adhesion vessels to stimulate aggregate formation. The cells were seeded in minimal medium with a defined serum replacement to reduce variability during differentiation. Free-floating aggregates were then embedded in the extracellular matrix mixture, Matrigel, that polymerised at room temperature and enveloped the stem cell aggregates. This initiates retinal differentiation in the aggregate and is therefore crucial for the process. The product Matrigel is an animal product with high batch-to-batch variability. The main components are collagen, laminin, fibronectin, entactin, and heparan sulphate (Kleinman et al. 1982; Xu et al. 2001). Matrigel contains several hundred peptides and proteins, making it an extremely complex matrix compound (C. S. Hughes, Postovit, and Lajoie 2010). The problems with this product are well known, but yet no efficient way to replace it could be developed. As the exact composition remains elusive in every new batch of Matrigel, it has to be evaluated according to its potential to induce retinal fate in stem cells. The only thing that can be influenced is the total protein concentration, which should be around 9.5 mg/ml or above (Eiraku and Sasai 2012). On D4, the cells formed aggregates with a clearly visible epithelium (Figure 12). Starting around D6, the expression of Rx-GFP started in the ROs, and thereafter, it increased consistently throughout the neuroepithelium in the following days. On D11, the Rx-GFP$^+$ areas are isolated manually using forceps. This requires practice and is heavily dependent of the person conducting the experiments. The size of the resulting Rx-GFP$^+$ fragments varies depending on factors like the quality of the aggregates and how the dissection is performed. Thereafter, the fragments were transferred into RMM2 with RA and taurine and formed roundish ROs that continued to grow. Until D14, the ROs were cultivated with the addition of taurine and RA, both of which increase the differentiation of retinal cell types, especially photoreceptors and RGCs (Forouzanfar et al. 2021; Huang et al. 2018). Taurine is an organic acid found in the retina that has been shown to increase rod photoreceptor differentiation (Altshuler et al. 1993; Militante and Lombardini 2002). Even though it has additional supportive functions in many processes, including oxidative stress, inflammation, and membrane stability, the withdrawal of the factor from the cultivation medium showed only minor changes in RO development (Eiraku et al. 2011; Menzie, Prentice, and Wu 2013; Kim and Cha 2014). RA, on the other hand, is an important factor in retinal development, and disturbance of the RA signalling *in vivo* causes severe ocular malfunctions (Isla-Magrané et al. 2022) In ROs it is known to promote photoreceptor differentiation (Kelley et al. 2020).

One of the most prominent differences between ROs that were generated using different protocols is the ability to form optic cup-like structures and a laminin layer on the outside of the ROs, which provides a basal lamina for the correct orientation of the apico-basal subdivision (Eiraku and Sasai 2012; Nakano et al. 2012; DiStefano et al. 2017). Something that could not be observed in our lab and is still challenging for many protocols (X. Zhong et al. 2014; Eastlake et al. 2019; Cowan et al. 2020; Völkner et al. 2021; Wagstaff et al. 2021). Matrigel provides basement membrane components for the formation of ROs but seemingly this is not sufficient to determine the basal side on the distal region and support optic cup formation.

Additionally, the formation of optic cups was mostly observed in protocols with varying oxygen levels at specific developmental timepoints. Changes in oxygen can stimulate progenitor cells and are involved in developmental processes (Simon and Keith 2008). This can be done by increasing the oxygen levels at D10 to 40% O_2 (Eiraku et al. 2011; Nakano et al. 2012). Another method includes

decreasing oxygen to 5% prior to D10 to create hypoxic conditions (DiStefano et al. 2017; H. Y. Chen, Kelley, and Swaroop 2020). Hypoxia is a physiological condition during early embryonic development that supports cell proliferation and morphogenesis. (Abdollahi et al. 2011). The preference of stem cells towards hypoxic conditions is also reflected by the stem cell niche of hematopoietic, mesenchymal, and neural stem cells, which range between 1% and 8% (Mohyeldin, Garzón-Muvdi, and Quiñones-Hinojosa 2010). Hypoxic conditions in the retina are caused by a lack of vasculature. It was thought that retinal astrocytes sense the hypoxic environment during retinal angiogenesis. But it turned out the the RPCs were the actual sensors of the hypoxic condition and that retinal astrocytes were influenced mainly by paracrine PEGF signalling and Tlx responding to HIF1 signalling from the RPCs (Tao and Zhang 2014). ROs lack vascularisation due to a lack of adjacent structures like the optic stalk, where the APCs and vascular endothelial cells emerge from. That leaves the ROs with hypoxia sensing RPCs that trigger HIF1 signalling. Thus, RO differentiation protocols that include varying oxygen concentrations could be beneficial for stem cells that prefer hypoxic environments or counteract hypoxia that triggers RPC signalling at later timepoints.

Following the protocol of Eiraku and colleagues did not result in the formation of optic cups. ROs showed an inverted phenotype with apical cells on the outside and basal cells on the inside of the optic vesicle-like structure (Figure 12). The ROs display a rather immature state of retinal development that could represent the neuroblast layer (Afanasyeva et al. 2021). Between P6 and P10 in mice, a transcriptional shift represents the functional maturation of retinal neurons *in vivo,* which includes the development of photoreceptor outer segments and outer plexiform layer formation that is missing in ROs (Brooks et al. 2019). This is in part due to missing signalling from the RPE but also to the limited maturation time. ROs derived from mouse stem cells start to lose structural organisation of the retinal epithelium, accompanied by cell death around approximately 30 days of cultivation (Eiraku et al. 2011; DiStefano et al. 2017; Brooks et al. 2019). Starting on D25, the ROs exhibited progressing loss of structure and thinning of the epithelium (Figures 13 F and 14 F). The development of ROs broadly resembles retinal *in vivo* development and shows similarities in the sequence of processes but is delayed in the expression of cell type specific genes (Brooks et al. 2019). Therefore, the neuroepithelium of ROs seems to remain pseudostratified and fails to form distinct layers as nuclei in the neuroepithelium are elongated and oriented along the apico-basal axis, while cells that are localising beneath the epithelium exhibit roundish nuclei (Figure 13 C). Although the layering is not as complex as it was reported in protocols using human-derived stem cells to differentiate ROs, mESC-derived ROs possess a retinal epithelium with apico-basal polarisation and defined regions containing preferred subgroups of retinal cells (Nakano et al. 2012).

Cell Type Distribution in the Neuroepithelium

RGCs are the first retinal neurons to arise. In ROs, RGCs make up a small portion of cells in the neuroepithelium, with approximately 6% on D18 and thereafter dropping below 4% on D21 and below 2% on D28 (Figure 15 A). This is in accordance with literature describing the mouse retina to contain 2-3% RGCs, which have their differentiation peak at E13 (Byerly and Blackshaw 2009).

In this work, RGCs were identified by their expression of Islet1 and localised on the basal side of the neuroepithelium at all timepoints assessed. This resembles the *in vivo* localisation of this cell type in the ganglion cell layer (Figures 13 and 15 B). With increasing cultivation time, RGCs remained consistently on the basal side (Figure 15 B). After exiting the cell cycle for differentiation, RGC nuclei migrate from the apical side to the basal region of the epithelium using their inherited basal process

or a newly extended process (Icha et al. 2016). The basal localisation of RGCs has an influence on the subsequent lamination of the retina and is, therefore, a rather robust process that is also conserved in ROs.

In the retina, the cells would be localised directly adjacent to the inner limiting membrane, the basal lamina that separates the retina from the vitreous body and is closely associated with MGC endfeet (Vecino et al. 2016). This limitation on the basal side is only seen in some ROs (Eiraku et al. 2011; Nakano et al. 2012; DiStefano et al. 2017). One of the main components of the basal lamina is Laminin α1 which is required for the polarization of RGCs. Laminin1 directs the orientation of axonal outgrowth of RGCs *in vivo* (Randlett et al. 2011). In Lam1 deficient retinas, the RGCs go through ectopic polarization and acquire stage 2 behaviour before axon extension. Stage 2 is a term used for the behaviour of neurons during a multipolar phase where they extend and retract short processes in multiple directions before projecting a single axon in the absence of polarising cues (Dotti, Sullivan, and Banker 1988). This phase also includes mislocalised centrosomes and centrosome wandering (Randlett et al. 2011). Many of the Islet1$^+$ cells migrate even further and localise deeper inside the RO, leaving the neuroepithelium (Figure 13). This could be a consequence of a missing basal membrane that directs the RGC migration. Additionally, these Islet1$^+$ cells exhibit a round nucleus in comparison to other nuclei that were stained inside the epithelium, which could indicate they did no longer possess the apical process and polarisation (Figure 13 B and C). It could also indicate that the neuroepithelium at this timepoint resembles the neuroblast layer that contains the photoreceptor progenitors, whereas the RGCs accumulate beneath, forming the presumptive ganglion cell layer (Nishida et al. 2003). This explains the measured decrease of RGCs over time (Figure 15 A).

Relative cell number measurements revealed the progressive loss of RGCs with increased cultivation times (Figure 15 A). This is a known problem that is directly connected to their function in the retina (Collin et al. 2019). During development, RGCs naturally undergo two waves of cell death that are characterised (Guerin et al. 2006). The first period is around E15 - E17 in mice, and the second one peaks between P2 and P5 (R. W. Young 1984). RGC death is associated with the deprivation of trophic factors. These are expressed in the retina and the primary visual centres and regulate survival, differentiation, and regeneration. RGCs form long axons that fasciculate to form the optic nerve that exits the retina via the optic stalk. Thereafter, at the chiasma, the optic nerves of both eyes merge. Some axons remain on the ipsilateral side while others cross the midline (Erskine et al. 2000). Information from the right half of the field of view continues in the left optic tract, while information from the left side of the field of view gets passed on by the left optic tract. The visual information reaches the optic tectum as a topographic projection and is then further processed (Stuermer 1988; Dütting, Handwerker, and Drescher 1999). When the axons fail to accurately project into their target structures, like the superior colliculus, the neurons will eventually die. This is a safety mechanism to ensure only the RGCs that formed the correct projections survive (Guerin et al. 2006). However, in ROs, this programmed cell death causes most of the RGCs to die since no optic nerve is formed and the axon connection to higher visual centres does not occur (Yamaguchi and Miura 2015). RGC specific genes were almost not detectable after D22 in ROs (Brooks et al. 2019).

Besides RGCs, some bipolar cells and a subset of amacrine cells are positive for Islet1 (Elshatory et al. 2007). They can be distinguished from the RGCs by the additional detection of Vsx2. The co-expressing cells are located inside the neuroepithelium at a more central position than the basally localised Iselt1$^+$ ganglion cells but only contribute to a minority of Islet1$^+$ cells (Figure 16). They account for 0.8% of total cells on D18 and D21, but since the Vsx2$^+$ cells are present throughout the whole epithelium, the orthogonal projection can falsely measure co-localisation. If Vsx2$^+$ and Islet1$^+$ cells are in close proximity or overlap in z direction, this could not be differentiated as separate cells in a three-dimensional space. The micrographs show that this is influencing the 28-day old ROs to a lesser degree,

where only a few distinct nuclei were stained positive for Vsx2 (Figure 16 B). Even though the evaluation of orthogonal projections is a potential source of errors, the evaluation method makes sense in the regard that the retinal cell types and mitotic cells were identified solely by the use of nuclear localised markers. Therefore, the quantitative comparison to the DAPI channel is a useful standard. In contrast to nuclear markers, cell plasma or membrane-associated marker proteins would overlap with cells in close proximity and resemble the cell numbers only poorly. The amount of signal of the stained cells would vary a lot for the area of one cell. The variability in nucleus size is lower compared to that and therefore better resembles the actual number of stained cells. The measurements were kept as comparable as possible by following defined parameters a give a useful tendency for the relative cell numbers inside the ROs.

$Vsx2^+$ cells are mainly RPCs due to the immature character of the ROs. Later in development, Vsx2 maintains expressed in bipolar cells, which are one of the last cell types that occur *in vivo,* with their differentiation peak on P3 (Byerly and Blackshaw 2009). Therefore, it is likely that most of the $Vsx2^+$ cells during this work were rather RPCs than bipolar cells. At D18 and D21, the $Vsx2^+$ cells were broadly distributed in the central region of the neuroepithelium. The transition from RPCs to bipolar cells could not be assessed in the experiments, but the cells showed a time-dependant restriction of their nuclei to the basal side which could indicate changes caused by maturation or differentiation (Figure 13). RPCs contribute to more than a third of the total cells in the neuroepithelium of ROs at D18 to D21 (Figure 15 C). It could be possible that the RPCs differentiate and thereafter only bipolar cells remain $Vsx2^+$, which would explain the drastic decrease to approximately 7% at D28. This is in accordance with the literature, where bipolar cells make up about 10% of the retina (Byerly and Blackshaw 2009). Bipolar cell polarisation is accomplished by the sprouting of axons from their bidirectional processes, indicating the presence of a pseudostratified epithelium (Morgan et al. 2006). This was also seen in ROs, where $Vsx2^+$ nuclei displayed an elongated morphology, demonstrating their polarisation along the apico-basal axis (Figure 13 B and C).

Otx2 is used as a marker for photoreceptors and their direct progenitors in ROs. During retinal development, Otx2 is expressed in postmitotic neurons during the formation of the optic cup, which are mainly photoreceptors. The earliest positive nuclei for Otx2 *in vivo* were detected at E13, mostly located in the RPE, and at P6 in the INL (Baas et al. 2000). This is because Otx2 is transiently expressed in bipolar cells that lose Otx2 expression with progressing differentiation (C. Koike et al. 2007). Upon *in vitro* cultivation of E16 explants, the number of $Otx2^+$ cells drastically increased after three days and represented two thirds of the neuroepithelium, as it was observed in ROs (Baas et al. 2000). Immunohistochemical staining of ROs showed that, besides the RPCs, the majority of cells in the ROs are $Otx2^+$ postmitotic cells (Figure 17). This is accordance with the *in vivo* development where photoreceptors are the most abundant cell type of the murine retina, comprising approximately 70% of the cells (Byerly and Blackshaw 2009). In the ROs, which represent an immature state of retinal development, the $Otx2^+$ nuclei reside in the apical half of the neuroepithelium but are not completely restricted to the apical side as they would upon full maturation (Figure 14). During development, the photoreceptors undergo bilateral migration in the *in vivo* retina so that mitotic cells can divide at the apical side (Rocha-Martins et al. 2023). Therefore, during development, many immature photoreceptors are in motion in this region but will migrate back to the apical side to fully mature. This is also in accordance with the pH3 staining showing the presence of apical mitotic cells (Figure 14). Even though the staining alone cannot validate bilateral photoreceptor migration, it is likely that the same mechanisms apply in this *in vitro* model. On D28, the preference of the $Otx2^+$ cells for the apical side is decreased, and the cells are more distributed in the central region of the epithelium (Figure 17). This is most likely due to the degeneration of the ROs. The epithelium of ROs starts to develop irregularities after 25 days in culture that are accompanied by the decrease of $Islet1^+$ and $Vsx2^+$ cells

that thereby influence the distribution pattern. The cell numbers for $Otx2^+$ cells remain high over time, supporting their survival upon long-term cultivation (Figure 17 A).

To get a better understanding of the dynamics of cell division inside the neuroepithelium of ROs, mitotic cells were immunostained. During mitosis, the phosphorylation of histone 3 at Ser^{10} is linked to chromosome condensation and is required for proper chromosome segregation (Wei et al. 1999; Crosio et al. 2002). This mechanism is conserved among vertebrates and can be used as a mitotic marker for cells in the late G2 phase and during mitosis (Prigent and Dimitrov 2003). ROs resemble the retina in regard to their cell types and their organisation in the apico-basal direction. Using an antibody against pH3, the nuclei of mitotic cells in ROs can be observed. Mitotic cells were restricted to the apical side of the neuroepithelium (Figure 14). This localization pattern, as well as the positioning of other cell types like the photoreceptors on the apical side and the ganglion cells basally, is in accordance with literature (X. Zhong et al. 2014). This means not only that proliferating cells divide apically but also that neurons like RGCs undergo migration afterwards that resembles the *in vivo* patterning mechanism of the retina. Here, pseudostratified cells are connected to the apical surface via actin and the Crumbs complex, which consists of Cdc42, Par3, Par6, and aPKCs (Randlett, Norden, and Harris 2011). Misexpression or loss of these components leads to detachment of mitotic cells from the apical side, resulting in ectopic division in the retina and loss of polarity of the tissue (Cappello et al. 2006; Costa et al. 2008). Mitotic cells showed no signs of ectopic division and remained closely restricted to the apical surface at all measured timepoints (Figure 17 C and D). Therefore, it is most likely that ROs use interkinetic nuclear migration to control their RPC pool, which was already observed in other ROs (Nakano et al. 2012).

MGCs are the last cell type to arise during retinal development (C. Cepko 2014). They are the single glial cell type to be generated from the same RPC pool as the retinal neurons (Turner and Cepko 1987). Their differentiation from RPCs is mainly regulated by a combination of the upregulation of Notch that causes the expression of bHLH genes, thereby inhibiting the expression of proneural bHLH genes. As Notch inhibits the potential for neuronal differentiation, the upregulation of Sox9 and, thereafter, NFIa leads to the gliogenic switch that induces MGC differentiation (P. Kang et al. 2012). This is crucial for gliogenesis not only in the retina but also in other parts of the CNS (Deneen et al. 2006). The prerequisite for this transition is the progressing transition of RPCs through the competence states until late retinal progenitors are generated. Late RPCs show a high degree of transcriptional overlap with MGCs (Blackshaw et al. 2004; Roesch et al. 2008). MGC-enriched genes are already upregulated in the late retinal progenitor population and remain expressed in mature MGCs, indicating a fluent transition between these cell types. This characteristic makes it difficult to distinguish between them.

Examples include Sox9 and NFIa, which can therefore be used as markers for late retinal progenitor cells that can differentiate into MGCs. These late RPCs were present in ROs starting between D18, where they were absent, and D21 where both factors could be detected (Figure 18 and Supplementary Figure 2). Sox9 and NFIa expressing cells are localised on the basal side of the neuroepithelium, which is in accordance with the positioning of the late $Vsx2^+$ cells that could represent bipolar cells. Both bipolar cells and MGCs reside in the same layer of the retina. NFIa is expressed in bipolar cells that were generated from the same late RPC pool and, therefore, does not exclusively mark MGC nuclei (El-Hodiri et al. 2022). Nevertheless, it is required for the differentiation of MGCs and remains expressed thereafter. Additionally, Sox9 was used, which is expressed upstream of NFIa but gets downregulated in differentiating postmitotic neurons (Poché et al. 2008). Therefore, the combination gives an impression if late retinal cell types are present and have the potential to differentiate into MGCs.

Identification of mature MGCs is often accomplished by their specialised morphology in combination with specific marker protein expression (Vecino et al. 2016). These markers are often directly

connected to cell functions in the tissue and are, therefore, expressed only late in MGC maturation. This includes marker proteins like the water channel Aqp4 or the potassium channel Kir4.1 (E. Newman and Reichenbach 1996; Nagelhus et al. 1998).

The maturation of MGCs takes a long time, and in human ROs, it requires at least 100 days of cultivation (Eastlake et al. 2019). The developmental processes in the mouse model only take a fraction of the maturation time of human ROs, but often ROs start to degenerate before developing the complexity of mature tissue. This is especially a limitation for the development of MGCs. Nevertheless, the differentiation of MGCs in murine ROs has been shown previously (Eiraku et al. 2011; Völkner et al. 2021). The differentiation of MGCs *in vivo* starts around E18 and peaks around P3 (Byerly and Blackshaw 2009). In RO on D20, MGC endfeet were detectable (Eiraku et al. 2011). Others show MGC differentiation in ROs starts around D22 (Brooks et al. 2019).

Many MGC marker proteins, however, are also found in other glial cells and, thus, do not exclude the possibility of co-staining with astrocytes or microglia. GFAP is a general glia marker often used for the distinction between neurons and glia. While this marker can be used for most astrocytes and microglia that are always positive for GFAP, MGCs do not express GFAP under physiological conditions (Lukowski et al. 2019). However, GFAP gets upregulated in MGCs during reactive gliosis, making it difficult to distinguish between glia cell types using only this one marker. A direct differentiation protocol for retinal glia was designed using Notch ligands to accelerate MGC differentiation (Chung et al. 2019). There, it was reported that the cells showed high expression of GFAP after the treatment. The expression decreased weeks after the treatment, but the authors could not clarify whether this was due to the maturation of MGCs that normally do not express GFAP or if the generated immature glial cells represent an astrocyte population. GFAP can only rarely be detected in healthy MGC endfeet, but not to the extent that the cells were clearly identifiable by the staining because it co-localises with the $GFAP^+$ retinal astrocytes in the tissue.

In ROs, $GFAP^+$ cells were found around D20 in the $Rx\text{-}GFP^-$ areas adjacent to the neuroepithelial region (Figure 18). The $Rx\text{-}GFP^-$ tissue was not further analysed as these cells were by-products of unknown origin and did not resemble retinal tissue. Inside the neuroepithelium, $GFAP^+$ cells occurred rarely and only late during cultivation around D28 (Figure 18). The cells showed a branched morphology, and some were oriented along the apico-basal axis of the epithelium. This indicates either the presence of MGCs undergoing reactive gliosis or the presence of astrocytes and was also observed in other protocols (Völkner et al. 2021). As retinal astrocytes require extraocular tissue for their generation and migrate into the retina, they should not be present in the ROs (Tao and Zhang 2014). Therefore, further characterization of the $GFAP^+$ glia was needed.

Since astrocytes have multiple overlapping tasks with MGCs, they also share the expression of many marker proteins (Roesch et al. 2008; Vecino et al. 2016). One of the most reliable markers to detect solely MGCs is CRALBP. Its specificity was confirmed in transgenic mice that express GFP under the control of the CRALBP promotor (Vázquez-Chona, Clark, and Levine 2009). The protein is involved in the visual cycle of rod and cone photoreceptors responsible for the regeneration of 11-*cis*-retinal after photoisomerization (Saari and Crabb 2005). Therefore, it is expressed mainly in the RPE that is adjacent to the outer segments but also throughout the entire cell body of MGCs (Bunt-Milam and Saari 1983; Muniz et al. 2006). Astrocytes do not express the gene as they are functionally not involved with visual pigment regeneration. Cralbp is only transiently expressed in immature astrocytes, but its expression is lost before reaching postnatal week two (Johnson et al. 1997).

ROs did not show any signal for the marker CRALBP at all times, indicating that no mature MGCs were present until D28 (Figure 18). Even in the ROs that displayed $GFAP^+$ cells, no CRALBP was detected, meaning the glial cells present in ROs most likely represent astrocytes rather than MGCs. To exclude

the possibility of a methodical problem, eyes from P28 WT mice were sectioned and used as a positive control. The retina displayed MGCs with Sox9$^+$ nuclei in the INL with bidirectional CRALBP$^+$ and vimentin$^+$ processes (Supplementary Figure 1). The eyes were treated exactly as ROs, and the same staining procedure was used, demonstrating that the lack of signal in ROs is due to the absence of the protein. This leaves two options: The cells could either need additional time to fully mature and express the protein, or MGC differentiation is not supported under the conditions of the RO differentiation protocol. To test this, ROs were cultivated for an extended period to provide enough time for maturation. But even after 40 and 50 days of cultivation, no expression of CRALBP could be detected (Supplementary Figure 1 B and C).

The characterization of ROs showed the formation of a polarised Rx-GFP$^+$ neuroepithelium that can be maintained for 25 days. The presence of basic retinal neurons and their positioning in the tissue according to the *in vivo* organisation of the retina were demonstrated. Glial cells were mainly absent and rarely occurring GFAP$^+$ cells were a by-product and identified as astrocytes. Since the development of CRALBP$^+$ MGCs did not occur even after prolonged cultivation time, the cells seem to lack signalling cues to differentiate into MGCs. Thus, the literature was screened for potential factors to promote their differentiation.

Targeting Notch Signalling to Induce Müller Glia Differentiation in Retinal Organoids

The least invasive way to integrate glial cells into ROs is to stimulate their generation in the tissue. This method has the advantage that no dissociation is required, and the glia cells can directly position themselves according to the ROs architecture. Upon differentiation, they can support the RO via soluble factors and provide cell-cell contact-mediated signalling at the same time. Notch signalling was targeted to maintain the proliferative RPCs and impede the differentiation process during the treatment, maintaining a large number of late RPCs. These could subsequently become competent to differentiate into MGCs.

The Notch signalling pathway is involved in several developmental processes in embryogenesis, including the fate decision between a proliferative state and differentiation (Lewis 1998). Notch is a transmembrane receptor involved in intercellular signalling. Upon interaction with one of its ligands, Delta like or Jagged, the extracellular domain gets cleaved by ADAM-family metalloproteases (Bray 2006). Thereafter, the intracellular domain gets cleaved by γ-secretase activity, and the Notch intracellular domain (NICD) translocates into the nucleus, where it forms a complex and regulates the transcription of genes like Hes1 and Hes5 (Perron and Harris 2000). With few exceptions, soluble ligands activate Notch signalling (Hicks et al. 2002). Supplementation of DLL4 or Jagged1 in a soluble form is used in several experiments to trigger the signalling cascade of Notch receptor-displaying cells (Masuya et al. 2002; Klose et al. 2015).

The treatment of ROs with DLL4 and Jagged1 from D15 to D18 led to a strong decrease in Vsx2 expressing cells (Figure 20), which represent RPCs, bipolar cells, and a subset of MGCs (I. S. C. Liu et al. 1994; Rowan and Cepko 2004). However, it has to be noted that D18 in ROs could be too early for the development of mature MGCs. Normally, they occur in the postnatal retina during *in vivo* development. The onset of bipolar cell differentiation is simultaneous with MGC differentiation and starts shortly before birth, around E18 *in vivo* (Byerly and Blackshaw 2009). This indicated that the cells

that are influenced by the treatment are RPCs, not bipolar cells or glia. But what causes the premature differentiation of RGCs in this experiment?

Retinal progenitors pass through different states of competence to differentiate into retinal neurons in a timely regulated manner (C. Cepko 2014). All retinal progenitors hold the potential to differentiate at any timepoint of this transition throughout the states of competence. Without a regulator that preserves a population of proliferating RPCs, the transition into further competence state would not happen, and all progenitors would react according to external signalling cues and differentiate into retinal neurons. The variety of retinal cell types from one common precursor can, therefore, only be established by the controlled maintenance of some progenitors. A good example is the fate decision of the first differentiating RGCs. Notch determines the subdivision into the RPC pool that maintains progenitor fate in contrast to the RPCs that develop the competence to differentiate upon Shh and FGF signalling (Austin et al. 1995). Thereafter, neural precursors produce the Notch ligand Delta so that intercellular signalling of Notch prevents neural fate specification in neighbouring cells (Henrique et al. 1995). The cells need this feedback mechanism since the presence of differentiated cell types can fuel further differentiation and diminish the progenitor pool.

Interkinetic nuclear migration provides a mechanism where cells can evade such cues and maintain a proliferating RPC pool. RPC nuclei localise at the apical side for mitosis, thereby avoiding the signalling cues they would experience more basally (Baye and Link 2008). A mathematical model demonstrated that the consequences of loss of interkinetic nuclear migration could be reduced capacity to produce later born neurons and decreased growth of the epithelium (Murciano et al. 2002).

A Notch signalling gradient in apico-basal direction is suggested to be responsible for the RPC fate decisions, with increased Delta in the basal region where neurons reside and the accumulation of Notch-expressing cells at the apical side (Del Bene et al. 2008). In zebrafish, a mutation in dynein1 causes the interkinetic nuclear migration to be altered. The movement in apical direction is diminished, but basal movement, which is associated with kinesin motor proteins, remains functional and leads to rapid and deep transport in the basal region of the tissue. As a result, mutant zebrafish show premature neurogenesis and exhibit low numbers or complete absence of MGCs and bipolar cells (Del Bene et al. 2008).

Since soluble Notch ligands are not restricted to one side or another, they disrupt the apico-basal gradient. The resulting ectopic activation of Notch signalling at the apical site could lead to the same results as predicted by the mathematical model or the mutant fish. The proliferating zone gets disturbed, and therefore, the cells exit the cell cycle and differentiate into the neurons of their current competence state. As described in the model, proliferating cells are missing, causing reductions in growth, and only a limited number of RPCs are left for the differentiation of later born cell types (Murciano et al. 2002). External addition of Notch does most likely not inhibit interkinetic nuclear migration, but it negates its effect. In ROs, bipolar cell generation peaked at D18, and MGCs could be detected at D20 (Eiraku et al. 2011). The treatment started prior to these timepoints and could, therefore, be more beneficial for the generation of rods that experienced their peak of differentiation earlier. The supplementation of the culture medium with taurine additionally stimulates rod differentiation in ROs. It was identified as one component of CM derived from retinal cells to increase *in vitro* rod specification (Altshuler et al. 1993). However, it also increases the differentiation rate of another late born cell type, which could “consume” many progenitors before they reach the state of competence required for MGC differentiation and should, therefore, be omitted. Thus, it was assumed that the loss of Vsx2 expressing cells is a result of the differentiation of RPCs into neurons that do not maintain Vsx2 expression upon differentiation, probably rod photoreceptors. Conversely, inhibition of Notch via soluble factors to induce photoreceptor differentiation is possible (Chew et al. 2022). DAPT

is a γ-secretase inhibitor, preventing the cleavage of the NICD and interrupting Notch signalling in the receptor expressing cell. Treatment of ROs with DAPT on D16 supported the differentiation of photoreceptors and decreased the $Vsx2^+$ proliferating RPC population on D18 (Eiraku et al. 2011). The inhibitor modulates the signalling, but in contrast to the soluble Notch ligands, it does not affect the receptor ligand gradient. Thus, seemingly contrary treatments result in the same differentiation pattern.

The timepoint and duration of the Notch ligand treatment could be essential factors influencing the differentiation pattern of retinal cell types. *In vivo,* this mechanism needs to be tightly regulated since small fluctuations can have a severe impact on the cellular composition of the tissue, potentially effecting its function. NFIa/b/x genes are associated with the late retinal progenitor state and its potential to differentiate into bipolar cells, or MGCs (El-Hodiri et al. 2022). Upon *in vivo* electroporation of P0 mice, the overexpression of NFIa/b/x caused an increase in bipolar cells and MGCs at P14 (de Melo and Blackshaw 2018; Clark et al. 2019). The overexpression is, therefore, not leading to solely glia differentiation but holds the potential to give rise to bipolar cells as well. These factors are closely associated with the gliogenic switch but are not efficiently suppressing the neural fate as needed for glia differentiation. Here, Notch signalling is required to repress neural differentiation via its downstream bHLH effector genes so that NFI factors can induce the glial fate. Notch is, therefore, not directly causing the glia fate determination but mediates RPC numbers and stabilises the competence state of the late retinal progenitors so that NFIa can act upon them (Androutsellis-Theotokis et al. 2006). Immunohistochemical staining of ROs showed that Sox9 and NFIa expression is upregulated between D18 and D21, marking the transition between early and late RPCs (Figure 19) The treatment with Notch ligands was not beneficial for the transition of early to late RPC as it did not lead to earlier expression of NFIa (Supplementary Figure 2). NFIa expressing late RPCs could, therefore, be the prerequisite for Notch-induced MGC differentiation in ROs and would require treatment on D21 or later. Under these circumstances, the glial fate should be favoured as a result of Notch activation.

Literature provides insights that the induction of MGC differentiation via Notch signalling is possible *in vivo* and *in vitro* (Furukawa et al. 2000). For example, retinal explants of P0 rat pubs were transfected with a virus construct leading to the expression of the NICD, simulating consecutive activation of Notch in the transfected cells. After 10 days of cultivation, 90% - 95% of transfected cells expressed the MGC markers CRALBP and CyclinD3 (Furukawa et al. 2000). This method indicates that exposure to Notch signalling over several days has no negative effect on MGC differentiation. Another study even suggested that postmitotic Notch expression is needed to maintain MGC fate while other gliogenic signals are upregulated, stabilising glia identity, and demonstrated that Notch signalling remains high over four to five days after the cell-cycle exit (Nelson et al. 2011). It is known that the expression of Sox9, NFIa, and Notch is maintained in mature MGCs (Clark et al. 2019). Ending the treatment before the MGC fate is stable could lead to the loss of the adapted glia fate. Suppression of Notch signalling in adult MGCs leads to the conversion into bipolar and amacrine cells, and additional suppression of the NFI factors causes a robust conversion of nearly all MGCs into neurons (Le et al. 2023). Blocking only the NFI factors in mice also resulted in the acquisition of neuronal competence (Hoang et al. 2020). Thus, prolonging the treatment with the Notch ligands could be an additional way to help stabilise the glial fate.

The relative cell numbers of $Islet1^+$ cells were not altered significantly by the treatment with DLL4 and Jagged1 compared to the untreated ROs (Figure 20). RGCs, which are mainly represented by this group, differentiate prior to the onset of the treatment on D15. The cell-cycle exit of RGCs peaks at E13 in mice and around D10 in ROs (Byerly and Blackshaw 2009; Eiraku et al. 2011). Therefore, the culture conditions between both samples were similar during RGC differentiation, which is represented in the cell numbers.

Nevertheless, it is important to mention that an increased differentiation of MGCs inside the ROs will be at the expense of other neurons (Clark et al. 2019). Therefore, the structure will not necessarily be more diverse, and alternative approaches need to be investigated to generate a three-dimensional cultivation system for retinal neurons that contains glial cells.

Co-Cultivation of Retinal Organoids with Primary Astrocytes

Glia cells modulate the microenvironment in the CNS and support neurons in many ways to maintain neuronal function (Vecino et al. 2016). The generated ROs showed no signs of mature MGCs at any timepoint assessed, and their differentiation in ROs could not be favoured. Another method to compensate for the missing support cells is the co-cultivation of ROs with glia cells from an external source.

Isolation of Primary Glial Cells

In addition to the lack of mature MGCs in ROs, obtaining mature MGCs for experiments can be a challenging task. MGCs appear omnipresent in the retina due to their morphology, which is characterised by long processes that envelop neuron nuclei and blood vessels. However, only 3% of the retinal cells are MGCs (Byerly and Blackshaw 2009). It is possible to isolate them from dissociated retinal tissue, but isolation protocols still need improvement. It was shown that primary human MGCs can successfully be isolated and maintained over multiple passages while remaining $CRALBP^{+}$ and without undergoing morphological changes (Y. Chen et al. 2021). But this is rather the exception, and, especially human primary cultures, come with additional problems like ethnic and safety concerns. As an alternative, ROs can also serve as a source of MGCs. But while mouse-derived ROs represent premature retinal tissue due to their limited cultivation time, human stem cell-derived ROs need tremendously increased cultivation times to generate mature MGCs. Isolation of glial cells between D70 and D90 yields cells that show transcriptomic and morphological features of MGCs, but the ROs were sometimes cultured for up to 300 days to ensure MGC maturity (Eastlake et al. 2019). This is consistent with the findings that in hESC-derived ROs, it takes four to five months to generate MGCs (X. Zhong et al. 2014). Cells that show the transcriptomic characteristics of MGCs as well as RPCs can be isolated earlier but do not represent the mature and functional MGCs that would be required for co-cultivation experiments (Eastlake et al. 2023).

A direct differentiation protocol in which RPCs were treated with Notch ligands resulted in heavily $GFAP^{+}$ cells that showed only a low increase in MGC-specific markers like GS, CRALBP, or Vimentin, which led the authors to conclude that the generated cells could potentially be astrocytes (Chung et al. 2019). Additionally, the cells decreased GFAP expression as well as Notch downstream effector genes like Hes1 and Hes5 six weeks after the treatment. As previously shown, MGCs are able to convert into neurons when they lack the repression of proneural bHLH genes by Notch signalling (Le et al. 2023). The loss or alteration of MGC gene expression was also reported for isolated MGCs from pig retina (Hauck, Suppmann, and Ueffing 2003; Merl et al. 2012). The expression of markers that are connected to MGC functionality, like CRALBP and GS, was maintained only three days after the isolation and, thereafter, declined drastically while cytoskeleton-associated proteins were upregulated accompanied by morphological changes (Hauck, Suppmann, and Ueffing 2003).

In 2002, the first human MGC line, MIO-M1, was characterised, which could potentially serve as a reliable source of MGCs (Limb et al. 2002). The cells showed morphological similarities to primary glia and maintained expression of marker proteins including GS, EGFR, CRALBP, and Vimentin even after 45 passages. Although this seemed promising, further analysis showed that the cell line simultaneously expressed markers for postmitotic neurons like opsins as well as RPC markers including Nestin and Pax6 and showed increased expression of the glioma-related gene thymosin beta 4 (Lawrence et al. 2007; Hollborn et al. 2011; Lukowski et al. 2019). Transcriptome analysis showed that the MIO-M1 cells formed a distinct cluster from all retinal cell types in the study, exhibiting a closer relation to astrocytes than MGCs (Lukowski et al. 2019).These findings further underlined the potential negative effects of long-term *in vitro* cultivation of these cells.

Taken together MGCs are highly specialised cells that require long periods of cultivation for their proper development; they are not easily generated *in vitro* and can be hard to maintain outside their preferred *in vivo* environment after isolation from primary tissue. Thus, they were excluded as candidates for the co-cultivation approaches with ROs.

Astrocytes, on the other hand, share many functions with MGCs. They are involved in the formation of the blood-retina barrier, ensheathment of axons, regulation of water homeostasis via aquaporins, waste product clearance, potassium buffering, and calcium signalling. They secrete trophic factors, are involved in glucose metabolism, and provide glutamine like MGCs (Fields and Stevens-Graham 2002; Allaman, Bélanger, and Magistretti 2011; Tao and Zhang 2014; Vecino et al. 2016). This is in accordance with transcriptome analysis of retinal cells that showed shared marker expression and a close correlation between retinal astrocytes and MGCs (Lukowski et al. 2019). Another important advantage is that astrocytes have a physiological niche in the GCL of the retina and are not completely artificial in the tissue. Therefore, it was decided to isolate astrocytes for the co-cultivation experiments.

Previously, a protocol for the isolation of cortical astrocytes from mice was established (Schildge et al. 2013). The preparation of four mouse pubs between P0 and P4 yields two T75 flasks of mature astrocytes. One week after isolation, the astrocytes should form a confluent monolayer with smaller cells on top. These are oligodendrocyte precursor cells and microglia that are removed by shaking the flask subsequently (Figure 20 B and C). However, this procedure worked only to a limited extent, as the cells would sometimes not detach regardless of the shaking intensity or duration. In this case, the cells were washed with PBS and shortly treated with trypsin to detach only the cells on top of the astrocyte monolayer. In most cases, a small number of astrocytes were lost during this process, decreasing the yield from one preparation. Additionally, the yielded cell numbers varied depending on the age of the sacrificed mice.

The most important step in the protocol is the attachment of the astrocytes after dissociation. The cells need to be at a certain density to form a monolayer in less than a week so that they can be purified according to the protocol and passaged afterwards. It is recommended that the cells reach confluency ten days after dissociation (Gottschling et al. 2016). Shortly thereafter, the astrocytes stop to proliferate and cannot be further expanded by passaging. Thus, improvements could be adapted from other glia isolation protocols. The simplest approach to yielding more cells was to increase the number of sacrificed animals for one isolation, as reported in another protocol that recommends five to ten pubs (Gong 2012). Nevertheless, in most cases, the number of available animals was limited, and therefore, it was essential to maximise the efficiency of the procedure to generate more outcome. In rare cases, no cells were attached to the flasks, but it was not clear whether this was due to complications with the coating of the flasks or cell survival during the dissociation process. Both are crucial steps to assure that the isolated astrocytes reach confluency. An isolation protocol for MGCs showed increased attachment to the surface for a combination of Poly-D-lysin and laminin coating

instead of Poly-D-lysin alone and could additionally be increased by dissociation using papain instead of trypsin (Pereiro et al. 2020). These could be beneficial remarks for the protocol used in this work.

Before the astrocytes could be used for experiments, they needed to mature. It was recommended to use the astrocytes for experiments after 21 days in culture (Schildge et al. 2013). The isolated astrocytes, which exhibited a spreaded morphology and contained big nuclei as described in literature, were checked for potential contamination of fibroblasts and stained for specific glia marker proteins to ensure astrocyte identity and maturity (Galland et al. 2019). The isolated cells strongly expressed the intermediate filament GFAP, which is indicative of glial cells. Additionally, expression of astrocyte markers like the water channel Aqp4 on their surface and the nuclear localised factors Sox2, $p27^{kip1}$, as well as Sox9 and NFIa, which are crucial factors for glia differentiation, was verified (Figure 21, Supplementary Figure 3) (Molofsky et al. 2012).

Co-Cultivation Setup

The method for the co-cultivation of distinct cell types heavily depends on the cell types and the research question. Glia and neurons are often co-cultured to investigate, for example, glia injury responses and find new neuroprotective factors with potential medical relevance for retinal disease models (Kitano, Morgan, and Caprioli 1996; Tezel and Wax 2000; Unterlauft et al. 2012).

In some cases, directly seeding both cell types onto 2D substrates is fully sufficient to get new insights (Hasel et al. 2017). This straight-forward approach can be enough to address some questions, but it has the drawback that the cells, once mixed, can hardly be separated afterwards for analysis. Transwell inserts are often used for the co-cultivation of cells that do not require direct contact but need the exchange of soluble factors for maturation or survival (Gottschling et al. 2016; Ulc et al. 2017). The cells are cultured in a shared medium and can have bidirectional exchange via soluble factors, but direct contact is not possible. The cells are separated by a membrane, with one cell type growing in the insert and the other one growing on the bottom of the dish. This can also be achieved by seeding the cells on two glass coverslips that are then cultured in the same culture dish with shared medium (Unterlauft et al. 2012). Over time, the co-cultivation setups got increasingly complex and were adjusted to the needs of specific research areas.

One of the biggest advantages of ROs is the self-autonomous differentiation of various cell types and the patterning of the tissue (Eiraku et al. 2011). The organisation in layers, or at least the subdivision in apico-basal regions inside the neuroepithelium, has a high degree of complexity compared to the plain 2D co-cultivation setups, mostly consisting of monocultures. Therefore, the integrity of the ROs was kept unaltered. In an attempt to enrich the culture medium with beneficial factors, the ROs were co-cultivated as a whole on top of confluent astrocytes in a shared medium. Nevertheless, the ROs did very rarely attach to the astrocytes underneath them, and in this case, they were excluded from the experiments. Co-cultivation with the primary astrocytes did not negatively affect the RO morphology. Independent of the duration of the co-cultivation, ROs maintained a strong Rx-GFP signal and neuroepithelial organisation, indicating that the ROs did not suffer from the culture conditions (Figure 21).

The cultivation of free-floating ROs has additional advantages over adherent cultures. Not only are they easy to transfer into new dishes without the need to dissociate them from a substrate and disrupt their organisation. It is known that adherent cells that grow in colonies exhibit very different forces and conditions on the outer rim of a colony compared to the central part of the colony (Rosowski et

al. 2015). Free-floating organoids still experience differences depending on their positioning inside the RO, including a gradient of nutrients that decreases towards the central region (Qian, Song, and Ming 2019). But their spherical organisation decreases, for example, the degree to which mechanical forces influence the cells.

Another advantage of free-floating samples is their accessibility for evaluation methods like microscopy. Adherent cultures require the substrate to fulfil certain criteria, like transparency, and have clear limitations in thickness, which automatically result in difficulties handling the samples. The ROs can be sectioned into successive 20 µm thick slices, which allows them to represent the full depth of the tissue, avoiding limitations for microscopy due to the thickness of the sample.

Influence of Soluble Culture Medium Components

The effects of glial cells to promote RGC survival, neurite growth, and protection of RGCs against hypoxic and endotoxic damage were demonstrated many times under physiological conditions (Kitano, Morgan, and Caprioli 1996; Heidinger et al. 1999; García et al. 2002). Especially soluble factors are known to be secreted by glia cells that mediate neuron survival. It was demonstrated that PEDF from MGCs saved RGCs from ischemic cell death, and glia-derived GDNF has a neuroprotective effect on photoreceptors. In addition, they secrete important extracellular matrix proteins as well as BNDF, NGF, and other growth factors (Frasson et al. 1999; García et al. 2002; Unterlauft et al. 2012; Del Río et al. 2011; Vecino et al. 2016). Even though the exchange of soluble factors was enabled in the shared environment, the co-cultivation of ROs from D15 to D18 with primary astrocytes did not lead to significant changes in the relative cell numbers of retinal neurons (Figures 22 and 23).

The co-cultivation was performed in favour of the ROs, and therefore, their regular culture medium was used. RMM2 differs from Astro MM mainly in the basal medium, which is DMEM-F12 instead of DMEM, and the serum. For the cultivation of ROs, a pre-treated foetal serum was used that was specifically designed to support embryonic stem cell cultures. The astrocytes, on the other hand, were grown in bovine growth serum, which is a bovine calf serum supplemented with vitamins, amino acids, trace metals, and other small molecules to support cell growth. As both manufacturers did not state the procedure that was used to treat the serum or the supplemented substances, it was not possible to identify specific factors as a clear difference between the two medium supplements. However, the serum for the Astro MM was heat-inactivated prior to use.

In literature, co-cultivation of astrocytes and neurons is performed in a defined medium with a plethora of supplements, but experiments were consistently performed without the use of serum (Tezel and Wax 2000; Unterlauft et al. 2012; Gottschling et al. 2016; Ulc et al. 2017; Hasel et al. 2017). Serum is a rather undefined culture medium supplement. It is an animal product with batch-to-batch variety that contains components like hormones, immunoglobulins, trace elements, and growth factors, as well as many undefined factors in low concentrations (S. Liu et al. 2023). Human serum is to 99% composed of only 22 proteins, with albumin being the most abundant (Issaq, Xiao, and Veenstra 2007). Serum from different animals is not comparable in composition, as demonstrated for APCs that differentiated into varying portions into oligodendrocytes and astrocytes depending on the medium and serum used (Raff, Miller, and Noble 1983).

Generally, serum contains components that are actively excluded from the CNS by the blood-brain barrier or the blood-retina barrier. It is no physiological substance for cells, and especially primary cell cultures that were never exposed to serum before can react to the factor-rich substance. Primary

microglia, for example, severely decrease their phagocytotic capacity upon cultivation with serum-supplemented medium (Bohlen et al. 2017). Astrocytes, like other glia involved in the immune response, are generally cultivated in a medium with heat-inactivated serum to reduce active components. One potential target often issued are different complement factors that are present in the serum (Triglia and Linscott 1980). These factors are heat-sensitive and can form precipitates during heat inactivation (Soltis et al. 1979).

Heat inactivation at 56°C for 30 min is the standard procedure and is widely used to inactivate serum components or prevent contamination. The parameters need to be tightly regulated to assure the reproducibility of experiments. The quality of the serum can suffer from the procedure when high amounts of proteins denature. It was shown that heat inactivation of serum had an effect on cell attachment on plastic and glass surfaces, indicating the destruction of factors that facilitate cell adhesion (Giard 1987).

Astrocytes are very specialised cells that react to neuronal injuries like infections, neurodegeneration, or ischemia (Sofroniew and Vinters 2010). This reaction is called gliosis, and responses can range from changes in their molecular expression and morphology to glia scar formation (Allaman, Bélanger, and Magistretti 2011). Astrocytes get activated by many different trigger substances, including growth factors and cytokines like IL6, LIF, CNTF, TNFα, INFγ, Il1, Il10, TGFβ, FGF2, mediators of innate immunity like lipopolysaccharides and Toll-like receptor ligands, neurotransmitters including glutamate, small molecules released by injured cells, molecules of oxidative stress, and many more (Sofroniew 2009). The result is a graded response in relation to the severity of the injury, which is accompanied by hypertrophy and upregulation of GFAP in most cases. Thereafter, they modulate their expression profile and can secrete soluble mediators that influence both the innate and additive immune responses (Farina, Aloisi, and Meinl 2007). As seen in Figure 21, the astrocytes did not change their morphology, which is indicative that they did not enter a severe active state. Nevertheless, it could not be excluded that the cultivation in serum-supplemented medium had a negative effect on the astrocytes. The RO itself is another potential source of activating factors for the astrocytes since the shared culture medium enables bidirectional communication. In a previous study, treatment with CM from activated astrocytes did not promote survival of the treated neurons (Gaul and Lübbert 1992). Retinal glial cells under stress situations like hypoxia or hydrostatic pressure can also secrete factors like tumour necrosis factor-α (TNF-α) or nitric oxide that induce apoptosis in RGCs in a transwell co-cultivation setup (Tezel and Wax 2000). Decreased survival rates of RGCs in co-culture setups with retinal glia under hypoxic conditions were also observed in other studies, underscoring the contrary effects glial cells can mediate (Unterlauft et al. 2012).

After the co-cultivation with astrocytes, the ROs did not exhibit alterations in regard to the numbers of retinal neurons compared to the untreated ROs. The line measurements suggest the same findings. The distribution patterns for the untreated ROs and the co-cultivated ROs are closest to each other and slightly differ from the rest. This can be seen after three days of co-cultivation in the distribution of $Vsx2^+$ and $Otx2^+$ cells (Figure 24), but becomes more evident after six days of culture (Figures 25 F and 26 E). Thus, it was assumed that the co-cultivation with astrocytes did not influence the ROs under the conditions tested.

Soluble factors that are secreted under physiological conditions from the astrocytes can accumulate in the supernatant of the medium. The CM from a mature confluent monolayer of astrocytes was collected, and ROs were then treated with the CM to see whether the astrocytes secreted potential beneficial factors when not confronted with other cells and serum proteins. On D15, half of the RMM2 medium from ROs was discarded and replaced by the conditioned Astro MM. Cultivating the ROs with the CM for three days changed the relative cell numbers of all three neuronal marker proteins assessed

significantly. While RGC numbers increased, the portion of $Vsx2^+$ cells and the $Otx2^+$ cells decreased (Figures 22 and 23).

Due to the CM treatment, the media composition was altered, and two controls were performed to get further insights. First, ROs were cultivated solely in the Astro MM to see whether the effect of the CM is attributed to the medium composition or the conditioning of the supernatant. And second, ROs were cultivated in RMM2 that contained heat-inactivated serum to test if heat-sensitive factors in the serum account for changes in RO composition (HiPan condition). The ROs cultivated under these two conditions did not show significant differences from each other but from other conditions, indicating that serum inactivation is a driving factor in the phenotypes observed. The differences in the basal medium or the supplementation of RMM2 with N2 do not appear to have an influence as prominent as when the serum is inactivated. This was observed for all cell types.

The responses of the retinal cells are not uniform, and different cell types do not seem to follow the same reaction after the treatments, as seen by the increase of $Islet1^+$ cells and the decrease of $Otx2^+$ cells after treatment with the CM. Therefore, the underlaying mechanisms are more complex than the lack of growth-promoting factors in one condition or their presence in another. Therefore, it seems like at least the RGCs and the photoreceptors react to different cues in the medium. Since all cell numbers were represented as a portion of DAPI stained nuclei, severe changes in the epithelial organisation could have an impact on the distribution. This was not the case, as all ROs showed a similar morphology after the treatments, and sectioning showed comparable epithelium thickness (Figures 25 and 26).

The portion of $Islet1^+$ RGCs was increased after the treatment with CM, indicating a beneficial effect of the culture medium or the conditioning process compared to the control. As mentioned earlier, the RGC differentiation peak is around D10 in ROs and, therefore, prior to the co-cultivation or medium treatment (Eiraku et al. 2011). This means the treatment most likely did not influence the differentiation rate but modulated the survival of the cells. During normal development of the ROs, the cell numbers decline with increasing age (Figure 15 A). In both experiments where ROs were cultivated in medium with heat-inactivated serum, the cell numbers of $Islet1^+$ cells increased even further but did not differ from each other. This indicates that heat inactivation has a positive effect on the RGCs that is present after CM treatment, where half of the medium contained untreated serum but not as pronounced as in the samples with inactivated serum only (Figure 22 A).

Bovine serum contains high amounts of glutamate, which is an important neurotransmitter used by photoreceptors, bipolar cells, and RGCs, but has dose-dependent negative effects on neuron cultures (Z. C. Ye and Sontheimer 1998). Glutamate activates N-methyl-D-aspartate (NMDA) as well as the two channels KAIN and QUIS (Choi, Koh, and Peters 1988). This results in sodium influx, causing depolarization and excitatory swelling of the neurons after secondary influx. The NMDA receptor alone is responsible for Ca^{2+} influx, causing neuronal injury (Choi 1985). Increasing concentrations of either glutamate or NMDA have dose-dependent negative effect on cell survival (Kitano, Morgan, and Caprioli 1996). The concentrations of glutamate in bovine serum were analysed and found to reach an average concentration of approximately 1 mM (Z. C. Ye and Sontheimer 1998). Astro MM and RMM2 both contain 10% serum and would, therefore, contain about 100 µM glutamate from serum alone. Exposure of RGCs *in vitro* to 100 µM glutamate causes the survival rate of the treated cells to drop to 70% (Kitano, Morgan, and Caprioli 1996). Another study even measured a decrease to 55% (Pang et al. 2007). The N2 supplement in RMM2 medium contains human insulin, which is added to protect neurons from oxygen-glucose deprivation-induced cell death (Mielke, Taghibiglou, and Wang 2006). But insulin also promotes the exocytosis of NMDAR to the cell membrane of neurons, increasing channel numbers on the surface (Skeberdis et al. 2001). This could even worsen the effect and explain

why both conditions using RMM2 showed the lowest numbers for RGCs. Since glutamate is added primarily through the addition of serum, the heat sensitivity of glutamate could cause the concentration to be low in the Astro MM and the HiPan conditions. The effect was still detectable in the CM approach, where half of the serum was untreated.

Glutamate-dependent toxicity can be avoided by the addition of an NMDA antagonist, co-cultivation on a monolayer of primary MGCs or PEDF treatment (Kitano, Morgan, and Caprioli 1996; Pang et al. 2007). Interestingly, primary astrocytes were already used to efficiently reduce glutamate levels of medium *in vitro,* reducing the glutamate levels in 3 h from 90 µM to less than 1 µM (Z.-C. Ye and Sontheimer 1998). This represents the extracellular concentration under physiological conditions (Nicholls and Attwell 1990). RGC survival rate was increased after co-cultivation with either MGCs or cortical astrocytes under hypoxic conditions or treatment with 200 µM glutamate (Kitano, Morgan, and Caprioli 1996). This is the case because astrocytes as well as MGCs are involved in glutamate uptake from their environment to protect neurons from cytotoxicity and to help regulate synapse sensitivity. Co-cultivation of astrocytes with neurons increased their sensitivity to glutamate, and it was suspected that this process is mediated by NMDA receptor subunit composition altered by the astrocytes (Daniels and Brown 2001). Glial cells have glutamate transporters like GLAST and GLT-1 (Chaudhry et al. 1995; Lehre, Davanger, and Danbolt 1997). The transfection of HeLa cells with GLT-1 showed that the cells were able to take up glutamate, and the process is dependent on external sodium and internal potassium (Pines et al. 1992). Additionally, it was shown that treatment with NMDA increases the expression of GLAST and GLT-1 in retinal glia (Furuya, Pan, and Kashiwagi 2012). The stimulation of the expression of more glutamate transporters could be a mechanism to counteract the toxicity and increase glutamate intake by glia cells.

The co-cultivation with primary astrocytes, however, did not show improved conditions for the maintenance of RGCs compared to the regular cultivated RGCs (Figure 22 A and B). This could be due to problems with the co-cultivation setup and the state of the astrocytes, but it also excludes glutamate as the factor responsible for the increased cell survival after cultivation with heat-inactivated serum. Nevertheless, it is exemplary for how a negative heat sensitive serum compound could affect RO composition. The distribution of Islet1$^+$ cells in the epithelium was additionally altered the most after the co-cultivation (Figures 24 and 25 E). In comparison to all other conditions, the apico-basal distribution was not as restricted to the basal side but displayed more cells in the central region. The curve, therefore, represents a more linear increase towards the apical side. The CM and the heat-inactivated serum conditions shared a similar distribution to the untreated control, displaying a sharp increase in the curve on the basal side (Figure 15 A and B). To check whether the shift is caused by an increased differentiation of Islet1$^+$ bipolar cells co-staining with Vsx2 was analysed. Double-stained bipolar cells would possibly be located in the central area, as seen for ROs on D28 (Figure 16). But the number of cells that were Islet1$^+$ and Vsx2$^+$ remained low and did most likely not interfere with the general tendency for the cell numbers and localisation of the cells inside the epithelium (Supplementary Figure 4).

In contrast to the increasing Islet1$^+$ cells, the Vsx2$^+$ cells showed an inverse effect. The untreated ROs showed the highest number of Vsx2$^+$ cells, and their portion decreased for all other conditions, independent of the duration of the treatment (Figure 22 C and D). The decrease of Vsx2$^+$ cells could be the result of the differentiation into postmitotic neurons from the proliferating RPC pool. D15 to D18 is too early for the differentiation into Vsx2$^+$ bipolar cells that could compensate for the decreasing cell numbers of Vsx2$^+$ RPCs. Even though it is assumed that heat inactivation of serum has only a minor negative effect on the growth of cultivated cells, these could depend on growth factors from the serum that promote their proliferative state (Giard 1987; Lee et al. 2022). After three days of co-cultivation or the different medium treatments, all Vsx2$^+$ cells were broadly distributed in the epithelium. The

curve of the line measurements is flattened and has its maximum in the central region of the epithelium (Figure 24 B). At this timepoint, there was no visual difference between the conditions. Prolonging the treatment led to small changes in the distribution of the VSx2$^+$ cells (Figure 25 F). The CM and the Astro MM conditions led to a shift of Vsx2$^+$ cells in the basal direction. On the apical side, the control ROs and the co-cultivated ROs additionally show a more flattened transition towards the apical side. The generated line measurement plots show a tendency and do not represent exact positioning changes. Nevertheless, it can be seen that the dataset for the ROs and the co-cultivated ROs show a similarity in their distribution that is different from the samples that were cultivated in heat-inactivated serum. The characterisation of the ROs showed that with increasing age, the Vsx2$^+$ cells position themselves further to the basal side, accompanied by a loss of cells (Figure 15 C and D). The same seems to happen in the ROs after cultivation in CM or Astro MM.

The number of pH3$^+$ mitotic cells was not altered by the experiments (Figure 23 C and D). After a treatment of three days, no significant changes in relative cell numbers were observable for all conditions tested. Only a small decrease in the CM condition was detected after six days of cultivation. Nevertheless, the number of mitotic cells ranged between 1% and 2% of the neuroepithelial cells under all conditions, making up a small portion with low variance. The only exception were the ROs at D28, which showed a decreased rate of mitosis (Figure 17 C). Their positioning in the epithelium did not change and remained restricted to the apical side, as seen in other ROs as well (Figures 24 D and 26 F) (X. Zhong et al. 2014).

Photoreceptors are the outermost neurons in the ROs and are, therefore, especially exposed to the medium compared to other neurons. Neuroprotection of photoreceptors by glia-derived factors was shown for many factors, including bFGF, CNTF, GDNF and BNDF signalling (Frasson et al. 1999; Wahlin et al. 2000; Zack 2000; Del Río et al. 2011). But again, co-cultivation with primary astrocytes did not show any effects on the Otx2$^+$ cell population (Figure 23 A and B). Interestingly, after treatment with CM, the number of photoreceptors drastically decreased. This was surprising since the cultivation of ROs in both media that were combined in the CM condition did not cause an effect this severe. It was excluded that the lower concentration of N2 supplement caused the decrease since the photoreceptors did differentiate normally in the HiPan condition that contained the same amount of N2. Growth in Astro MM did not reduce the photoreceptor numbers to that extent either.

After three days of cultivation, the control ROs were not significantly different in photoreceptor numbers than the ROs that were cultivated in Astro MM or HiPan. After six days, the ROs cultivated in Astro MM showed numbers for Otx2$^+$ cells that were significantly different from the untreated ROs and the CM treated ROs, placing them between those (Figures 23 A and B). It could be possible that the cortical astrocytes secrete negative factors when cultivated in Astro MM that were then enriched in the CM but not in RMM2 during the co-cultivation (Tezel and Wax 2000). But as a decrease in Otx2$^+$ cells can also be seen after the cultivation in unconditioned Astro MM, it is unlikely that the effect is solely induced by secreted factors from the astrocytes, even though the effect is prominent in the CM condition. The experiments did not influence the overall positioning of the cells in the epithelium. Regardless of the cell numbers, the positioning remained apical (Figures 24 C and 26 E). The distribution of the Otx2$^+$ cells in ROs and the co-cultivated ROs did not increase as sharply as the ROs treated with CM, the Astro MM, the HiPan medium after three days. The effect was still present after six days of treatment, showing differences between the samples cultivated in RMM2 and the other samples. Dose-dependent signalling from factors in the medium the astrocytes or the serum could be required for photoreceptor differentiation but did not alter the distribution in the epithelium. Nevertheless, the combinatory effects of several factors cannot be separated or identified by the experiments conducted.

The co-cultivation of ROs with primary astrocytes or their CM did not show beneficial effects on the cultivated ROs. The changes in neuron numbers and their distribution in the epithelium are most likely induced by one or more heat-sensitive factors that are denatured during the heat inactivation of the supplemented serum.

Cell Contact-Mediated Co-Cultivation

The soluble factor-mediated co-cultivation of ROs and primary astrocytes or treatment with their CM did not result in clear evidence for neuroprotective or otherwise beneficial effects. Thus, the setup was changed to a cell-cell contact-mediated approach that better resembles the multiple glia-neuron contacts of the *in vivo* retina. To introduce the isolated astrocytes in a shared environment, a new model system was established.

Reaggregation of Retinal Organoids

Retinal glia form a lot of cell contacts with their surrounding neurons. Especially MGCs that span all retinal layers are in direct contact with neurons of each type. The additional integration of a cell type into an already established system is challenging. RO-derived retinal cells and astrocytes need to be brought together in a shared culture system. While differentiated ROs need to be dissociated to access all cells, a complete loss of structure caused by the dissociation would not be desirable. To achieve this goal, the dissociation of ROs and their ability to reorganise themselves need to be analysed.

In contrast to MGCs, which directly grow in their physiological niche inside the retina, retinal astrocytes migrate into the tissue following RGC axons and hypoxia-induced signalling (Tao and Zhang 2014). Even though ROs show apico-basal polarity and organise themselves in a manner where the RGCs are located on the basal side of the neuroepithelium, resembling the *in vivo* situation, it is questionable whether retinal astrocytes could invade the RGC “layer” in ROs. Although similar approaches were followed to integrate microglia into cortical organoids (Wenzel et al. 2023). This can be done by the generation of microglia inside the cortical organoids or by adding them subsequently to cortical organoid generation (Abud et al. 2017; Ormel et al. 2018). For the latter, microglia were added to the culture medium that followed chemoattractant cues towards the cortical organoids and thereafter migrated into the tissue (Abud et al. 2017). This integration method does not require the dissociation of the organoid. This is due to the character of microglia, which are highly mobile and small in size. Microglia integration in midbrain organoids led to increased neuronal maturation, demonstrating the beneficial effect of glia neuron co-cultivation (Sabate-Soler et al. 2022). Astrocytes are bigger and do not follow the same migration pattern. They require guidance cues provided by the RGCs and their axons at a specific stage of development that ROs lack (Tao and Zhang 2014). The integration of the cortical astrocytes, therefore, needs the dissociation of the ROs.

After the dissociation of ROs, they were able to form new structured aggregates under low adhesion conditions called reaggregated ROs. Rx-GFP$^+$ cells formed aggregates that were disorganised at first but formed an epithelium over the first week (Supplementary Figure 5). They were cultivated for two weeks after reaggregation and showed the organisation of an Rx-GFP$^+$ polarised neuroepithelium (Figure 27). At this timepoint, they were comparable to ROs of the same age and showed a similar cell type composition (Figures 28 and 29). The epithelium showed the same apico-basal polarisation as in

the ROs, containing $Otx2^+$ photoreceptors on the outside and $Islet1^+$ RGCs on the inside. This demonstrated that reaggregated ROs were able to reproduce RO organisation to a high extent (Figures 28 and 29).

Like during early embryonic development, a symmetry-breaking event needs to take place in the structure to define the orientation of the established polarity. In single cultivated neurons, the first step to polarity is the extension of the axon (Craig and Banker 1994). Thereafter, reciprocal cAMP/cGMP signalling ensures the formation of a single axon. The upregulation of cAMP determines the axon and leads to cGMP upregulation in the other processes, causing them to become dendrites (Shelly et al. 2010). In the case of the ROs, the starting population of cells is heterogeneous. At the timepoint of dissociation on D14, the ROs already consist of RPCs and postmitotic neurons. One of the most prominent external cues is the medium accessibility, which could result in a nutrient and growth factor gradient that could define the apicobasal polarity.

Cell sorting mechanisms are regulated via cell-cell contact-mediated signalling between neighbouring cells, cell-matrix interactions, or soluble factors. During the specification of the optic field, for example, cells in the presumptive eye field sort themselves out according to autonomous Rx expression. It was shown that Rx^- cells were excluded and only Rx^+ cells contributed to the optic vesicle (Medina-Martinez et al. 2009). On D14, most of the cells in the ROs are still Rx^+ RPCs that could sort themselves and exclude Rx^- cells from the formation of the reaggregated ROs (Supplementary Figure 5). Thereafter, the aggregate grows quickly and produces new RPCs. Over the next three days, the reaggregated ROs show signs of neuroepithelium. It is not known whether postmitotic neurons are excluded from the process or if they are integrated and enveloped by the RPCs that structure their surroundings accordingly (Supplementary Figure 5). The underlying mechanism for cell contact-mediated sorting is the expression of membrane cues, as seen in the retinal plexiform layer, to constrain neurons from this region. The stratification of the retina is orchestrated by the interplay of adhesive and repulsive molecules to direct neurites (Matsuoka, Chivatakarn, et al. 2011). This includes homophilic cell adhesion molecules that direct synapse formation in the correct sublaminae of the IPL (Yamagata and Sanes 2008). Something also shown for the transmembrane Semaphorin6A signalling with the Plexin4A receptor (Matsuoka, Nguyen-Ba-Charvet, et al. 2011).

Many experiments in the past investigated the reaggregation of retinal cells in chick embryos, trying to understand the requirements of this process. Different methods were applied to study the interaction of the cells upon dissociation. In one approach, retinal cells were dissociated and subsequently embedded in a collagen gel (T. Watanabe and Raff 1990; Altshuler and Cepko 1992). Here, cell densities can be modified, and different maturity states of retinal cells were combined in mixed-age cultures (Belliveau and Cepko 1999). But in these experiments, the cells were seeded very sparsely, did not form three-dimensional clusters of tissue, and were, therefore, not comparable to the reaggregated ROs. The next step was three-dimensional pellet cultures from E15 rats, where retinal cells were dissociated, pelleted and transferred onto a polycarbonate filter. Thereafter, the cells were organised into two distinct types of neuronal rosettes that contained several cell types that were generated from RPCs *in vitro* (Takashi Watanabe et al. 1997). The same was demonstrated for the dissociated central retina from embryonic chick that was reaggregated by rotation culture. This resulted in the formation of aggregates with neural rosettes (P. G. Layer and Willbold 1989). The experiments show the intrinsic potential for cellular organisation, but they also indicate the requirement for additional cues, most likely extraretinal tissue that was removed prior to dissociation. When the chick retina was dissociated from the retinal margin with adjacent RPE cells, the aggregates formed one continuous epithelium with perfect lamination, including all retinal layers in the correct sequence with photoreceptors on the outside (P. G. Layer and Willbold 1989). This observation was repeatedly proven in varying setups, showing that layering also occurs after the co-cultivation of

dissociated retinal cells in the same medium as a monolayer of RPE cells (Rothermel et al. 1997). In contrast, aggregates from E5 and E6 chicks fail to form laminated aggregates in the absence of RPE. When they are co-cultivated with RPE, the organisation of aggregates from E6 and E7 retinae could not be rescued, and they formed neuronal rosettes. Retinal cells from E5, however, could form continuously laminated aggregates in the presence of RPE or its CM. It was concluded that the age of the retinal cells and soluble factors from the RPE are crucial for the formation of such highly organised aggregates. Similar experiments were performed with quills and zebrafish, indicating a conserved mechanism underlying the process (Paul G. Layer et al. 1990; Eldred, Muresan, and Harris 2017).

It was additionally suggested that the reorganisation is glia-mediated. Not only do MGCs share some of the secreted factors of RPE and could recreate the effect of cultivation with RPE, but MGC scaffold formation is required for the sorting of cells (Rothermel et al. 1997; Elmar Willbold et al. 2000). In the previously mentioned experiments with reaggregated retinal cells from chick embryos, immunostaining of MGCs showed their collective contribution to the epithelial organisation (Elmar Willbold et al. 2000). It was observed that MGCs formed organising centres after four days *in vitro*. With progressing cultivation, these fused and formed one structured aggregate with radial glia cells densely spanning the epithelium (Rothermel et al. 1997). These scaffolds of radial processes stabilise the surrounding cells in columns (E. Willbold et al. 1995).

Immunostaining of reaggregated ROs showed that no CRALBP$^+$ cells were present and the organisation of the epithelium was, therefore, not guided by MGC processes. The number of GFAP$^+$ glial cells was as low as in untreated ROs and did not show the required morphology to form the organising centres that lead to the structuring of the tissue. Additionally, astrocytes are not radial glia and, therefore, cannot provide a three-dimensional scaffold for the formation of a regionalized epithelium. As the reaggregated ROs were additionally not cultured in the presence of RPE, it is interesting to take a closer look at the secreted factors that could potentially support the lamination behaviour. Several factors, including bFGF, NGF, BNDF, taurine and RA were previously added to the cells in the absence of RPE but failed to recreate the level of organisation (Rothermel et al. 1997). Finally, the factor Wnt2b was identified as a laminar-inducing factor (Nakagawa et al. 2003). In the retina, Wnt2b is expressed in the ciliary margin zone and controls the proliferation of RPCs (Kubo, Takeichi, and Nakagawa 2003). Therefore, it would be interesting to further investigate if Wnt2b is present in the ROs and especially in the reaggregated ROs to guide cellular organisation.

Glia-Neuron Assembloids

As a next step, the isolated cortical astrocytes were integrated into the reaggregating ROs to create a supportive environment for the retinal neurons. The cells formed an aggregate that is, per definition, the combination of a regionalized neural organoid and a specialised cell type and, therefore, termed an assembloid (Pașca et al. 2022).

After the mixed seeding, the assembloids were cultivated for two weeks and, thereafter, analysed for their degree of organisation. Surprisingly, in all cases, the epithelial organisation was disrupted by the integration of primary astrocytes (Figure 30). This finding supports the idea that the organisation is mainly coordinated by cell-cell contact-mediated sorting of Rx-GFP$^+$ RPCs. Since the astrocytes are Rx$^-$ and additionally derived from a different genetic source, it is likely they were excluded from the sorting process. Microglia that were added from a different source and invaded cortical organoids died after approximately one month, while simultaneously generated microglia from the same background remained alive in the tissue (Wenzel et al. 2023). To this point, it is not yet clear whether many

astrocytes died during the cultivation. The incorporation of the astrocytes was confirmed by staining of GFAP, which showed expression in all assembloids (Figure 30). Nevertheless, they are mostly localised on the outer region of the assembloid, indicating that the attempt of the retinal cells to sort them out restricted them to the outer regions. As this is also the region where the epithelium would normally form, this could interfere with the process of reorganisation. Instead of one continuous epithelium, the assembloid contains many neuronal rosettes (Figure 30 D). Therefore, the retinal cells were still able to establish polarity, but not to the extent of forming an epithelium. In the rosettes, the central region consists of $Otx2^+$ cells and $pH3^+$ mitotic cells, identifying the region as an apical domain. Apical mitosis in the neural retina is a crucial and rather robust mechanism. In the retina, photoreceptors even undergo bidirectional migration to support apical mitosis by generating space (Rocha-Martins et al. 2023). Interkinetic nuclear migration has also been confirmed in ROs (Nakano et al. 2012). During interkinetic nuclear migration, the cells hold on to the inner limiting membrane as well as the outer limiting membrane. The pseudostratified cells are connected to the apical surface via actin and the Crumbs complex, which consists of Cdc42, Par3, Par6 and aPKCs (Randlett, Norden, and Harris 2011). Misexpression or loss of these components led to the detachment of mitotic cells from the apical side, resulting in ectopic division in the retina and loss of polarity of the tissue (Cappello et al. 2006; Costa et al. 2008). As the cells are capable of performing apical division in restricted areas, they could potentially be able to generate an organised epithelium if the surrounding environment provides a uniform cue.

The assembloids resemble the reaggregated retinal cells from embryonic chick. The aggregates of dissociated E6 and E7 retinae also formed neuronal rosettes with the photoreceptors in the centre (P. G. Layer and Willbold 1989; Rothermel et al. 1997). This changed, however, after the addition of soluble organising factors or providing a basal lamina-like structure. Complete retinal reorganisation of dissociated retinal cells from E6 chick embryos was possible after grafting onto the chorioallantoic membrane of embryonic chick (Fujisawa 1971). The retinal cells first formed neuronal rosettes that merged after the parts of the graft that were not in contact with the membrane were not supported. Thereafter, one neuroepithelial layer formed on the chorioallantoic membrane, with the GCL proximal to the membrane and the photoreceptors distal (Fujisawa 1971). The chorioallantoic membrane is a highly vascularized extraembryonic membrane (Nowak-Sliwinska, Segura, and Iruela-Arispe 2014). Vascularization of the neural retina is located mainly at the GCL which is why it is not surprising that the retinal cells used the membrane as a basal side and oriented the layering accordingly.

The idea to introduce an overarching cue that could rescue the disorganised phenotype of the assembloids was adapted for the assembloids. Therefore, an artificial basal side was established in the assembloids by the introduction of an aggregate of the primary astrocytes. This resembles the *in vivo* localisation of astrocytes in the retina and provides initial cues for the establishment of the apico-basal polarity of the retinal cells that were added in a second step. Astrocytes produce extra-cellular matrix proteins that could imitate the basal lamina. The importance of the basal lamina for RO morphogenesis and the orientation of the layering were shown before (Eiraku et al. 2011; Nakano et al. 2012; DiStefano et al. 2017). The basal lamina can provide orientation for proliferating cells in the assembloid. After mitosis, one cell inherits the basal process while the other cell forms a new basal process before the initialization of migration. This means at least the newly generated RPCs should be able to direct a basal process if they are able to identify the basal region (Baye and Link 2008). As interkinetic nuclear migration is still promoted inside the neuronal rosettes, this could increase the overall organisation. Additionally, the astrocytes provide soluble factors that could form a gradient and exhibit structural differences, including surface proteins and altered mechanical properties like lower stiffness and more elasticity (Vecino et al. 2016).

The integration of the astrocytes as a guiding element did not promote the assembloidal organisation, and no continuous neuroepithelium was formed (Figure 31). Regardless of the adjustments, the assembloids were similar to the ones with unstructured integration of astrocytes. They displayed cells that express Otx2, Islet1, Vsx2 and a small number of $pH3^+$ mitotic cells (Figure 31 B and C). There were some neuronal rosettes, but the cells were mainly spread throughout the aggregate without orientation. The numbers of $Islet1^+$ and $Vsx2^+$ cells were not measured as no epithelium was present, but the staining showed only a few immunoreactive nuclei on D28. Additionally, the $Islet1^+$ cells mostly displayed co-staining with Vsx2, showing the same tendency as for the ROs around D28, almost all RGCs were dead (Figure 31 D).

The nutrients and growth factors provided from the outside and cell or matrix contacts in the central region of the aggregate were not enough to direct the cells towards the formation of an epithelium. As the integration of the astrocytes was the only alteration to the reaggregation protocol, they are responsible for the disruption of the epithelium. For the mixed approach, it was suggested that the interference of the additional cell type with the cell-cell contact-mediated sorting causes the disruption. In the glia core assembloid, this does not seem to hold true. Even though most of the cells do not experience direct contact with the astrocytes, they still fail to organise. The outer region of the assembloid only contains the retinal cells, and the same sorting mechanism that guided the organisation of the reaggregated ROs without glia should apply. As this is not the case, the astrocytes probably introduce factors that inhibit the formation of an epithelium. As seen in many glial cells, isolated astrocytes change their expression profile during *in vitro* cultivation. This can be reduced by supplementation of factors cultivated under serum-free conditions (Foo et al. 2011). Co-cultivation with neurons can also positively influence astrocyte activity and metabolism, as they require many factors for homeostasis (Wenzel et al. 2023). In 2D co-culture setups where astrocytes were directly seeded on neurons, they developed a stellate morphology after nine days (Hasel et al. 2017). The fact that they need recovery time hints at the problem that glial cells react to injury to their tissue but are often isolated from tissue for experiments and dissociated for single cell analysis, therefore influencing their *ex vivo* gene expression (Marsh et al. 2022).

There were no methodical problems with the integration in general, as 12 out of 13 assembloids integrated the glia core in the central region, as demonstrated by immunostaining for GFAP (Figure 31 A). The glial cells survived the cultivation in the assembloids, as they are still present after 14 days of cultivation, but as the aggregate is densely packed with glia, it is not clear whether the astrocytes undergo reactive gliosis. The influence of serum has been discussed before, but since the assembloids were cultivated in RMM2 containing serum, the same problems apply as in the soluble factor-mediated approach. Glial cells change their expression after contact with serum and remain dysregulated or need long recovery times (Foo et al. 2011; Montilla et al. 2020). The serum proteins could mimic a disruption of the blood-brain barrier and, therefore, activate the glial cells (Zamanian et al. 2012).

Conclusion

In this work ROs, were generated and used for glia-neuron co-cultivation approaches with primary cortical astrocytes. The generated ROs had Rx-GFP$^+$ pseudostratified neuroepithelium with apico-basal polarity. RPCs underwent mitotic division on the apical surface, and postmitotic neurons positioned themselves according to the *in vivo* organisation of the retina. This included apically localised photoreceptors, basally localised RGCs and bipolar cells in the central to basal region of the epithelium, while MGCs remained absent. The treatment of the ROs between D15 and D18 with the soluble Notch ligands DLL4 and Jagged1 did neither increase the portion of late RPCs nor did it induce MGC differentiation. Therefore, it was necessary to design co-cultivation approaches targeting different mechanisms of action to compensate for the missing retinal glial cells.

Soluble factor-mediated signalling was established by the co-cultivation of ROs with primary astrocytes or their CM. The cultivation in shared retinal maturation medium for three or six days did not show a significant effect on ROs with regard to the relative cell numbers of the neurons and mitotic cells or their localisation in the epithelium. Additional unidirectional treatment of ROs with CM derived from astrocytes was performed to avoid the potential negative effect of the co-cultivation and the serum-supplemented medium. The treatment with CM resulted in altered cell numbers and minor shifts in the localisation tendencies of the retinal neurons. Control experiments performed with both media and heat inactivation of the serum led to comparable effects, indicating that the alterations were not caused by glia-mediated signalling but rather by the comminatory effects of culture medium components, especially the serum.

Cell-cell contact between retinal neurons and primary astrocytes was enabled by the generation of a new model system, reaggregated ROs. Dissociated ROs formed initially disorganised aggregates under low adhesion conditions that progressively reorganised themselves until they formed a continuous neuroepithelium after four days. The reaggregated ROs were comparable to the regular cultivated ROs of the same age. They comprised identical cell types, some of which even displayed increased cell numbers and re-established the apico-basal polarity and cell type localisation in the tissue.

The generation of assembloids by the integration of primary astrocytes into the reaggregated ROs disrupted the reorganisation of an epithelial structure, leading to the disorganised distribution of most retinal neurons. As the cells did not lose the capacity to form polarised neuronal rosettes with apical centres, it was suggested that contact-mediated cell sorting could be impaired by the addition of the astrocytes.

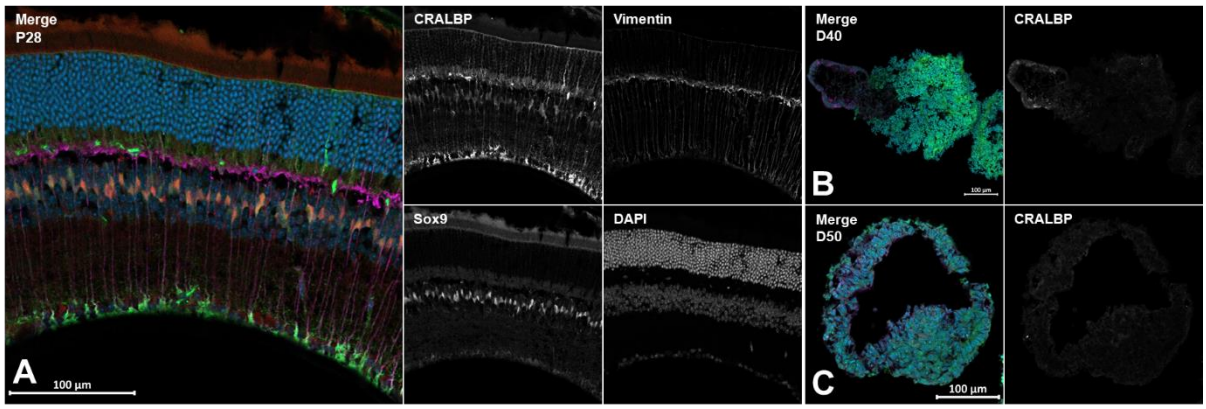
Providing overarching signalling cues to guide collective organisation of the retinal cells was attempted by the introduction of an artificial basal side. A glia aggregate was generated prior to the addition of the retinal cells, thereby integrating it in the central region of the assembloid, as confirmed by immunostaining of GFAP. The outer region of the assembloid comprised solely retinal neurons, but the establishment of a continuous neuroepithelium failed, and neuronal rosetted formed. Therefore, it was assumed that soluble factors from the astrocytes rather than contact-mediated signals were responsible for the loss of structure.

In summary, co-cultivation of ROs, or organoid-derived retinal cells, with primary astrocytes was addressed in this work but could not provide beneficial cues for improved RO cultivation. Nevertheless, the reaggregation of ROs is a promising model to investigate neuroepithelial formation and could give valuable insights into the complex processing underlaying retinal organisation and regeneration.

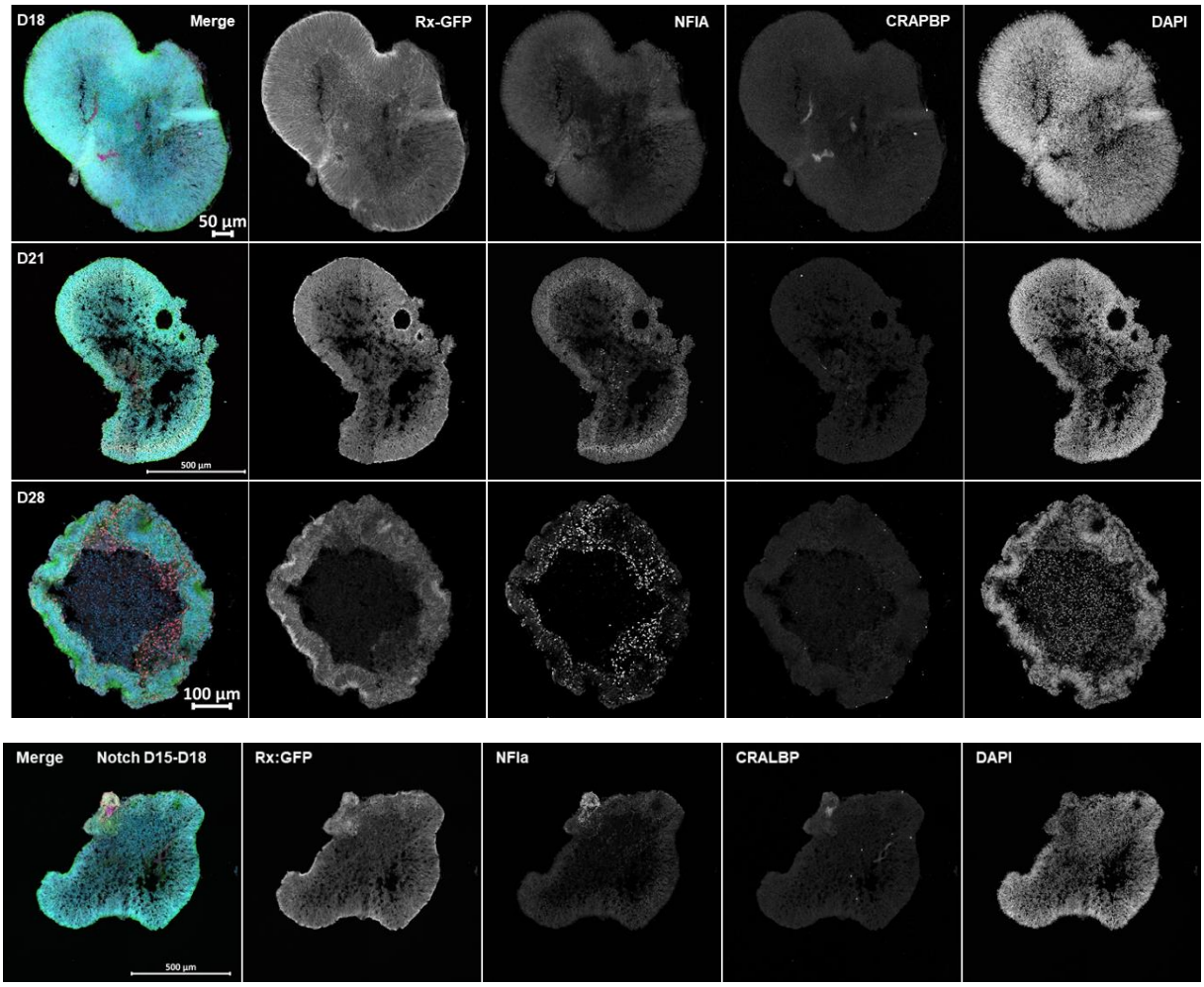
Table 6: Abbreviations

APC	Astrocyte precursor cells
bHLH	Basic helix-loop-helix
BNDF	Brain-derived neurotrophic factor
CM	Conditioned medium
CNS	Central nervous system
CRALBP	Cellular retinaldehyde binding protein
DLL4	Delta like ligand 4
DMEM	Dulbecco's modified eagle medium
E1	Embryonic day 1
EFTF	Eye field transcription factor
FCS	Foetal calf serum
bFGF	Basic fibroblast growth factor
GCL	Ganglion cell layer
GDNF	Glial derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HBSS	Hanks' balanced salt solution
HIF-1	Hypoxia incurable factor 1
INL	Inner nuclear layer
IPL	Inner plexiform layer
KSR	Knockout serum replacement
LIF	Leukaemia inhibitory factor
mESCs	Mouse embryonic stem cells
MM	Maintenance medium
NICD	Notch intracellular domain
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
O-2A progenitor	Oligodendrocyte-type-2 astrocyte progenitor
P1	Postnatal day 1
PBS	Phosphate buffered saline
PEDF	Pigment epithelium derived factor
PFA	Paraformaldehyde
PDGF	Platelet-derived growth factor
RA	Retinoic acid
RGCs	Retinal ganglion cells
RDM	Retinal differentiation medium
RO	Retinal organoid
ROI	Region of interest
RPCs	Retinal progenitor cells
RPE	Retinal pigment epithelium
RT	Room temperature
RMM1	Retinal maturation medium 1
RMM2	Retinal maturation medium 2
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor

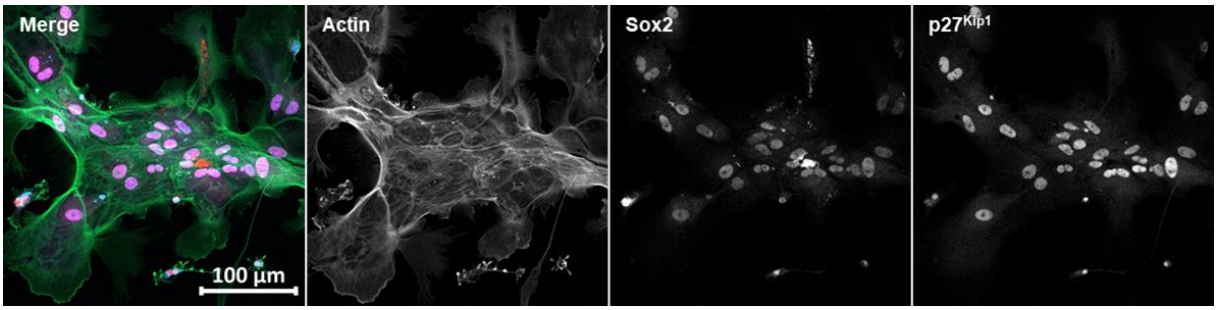
Supplementary Information



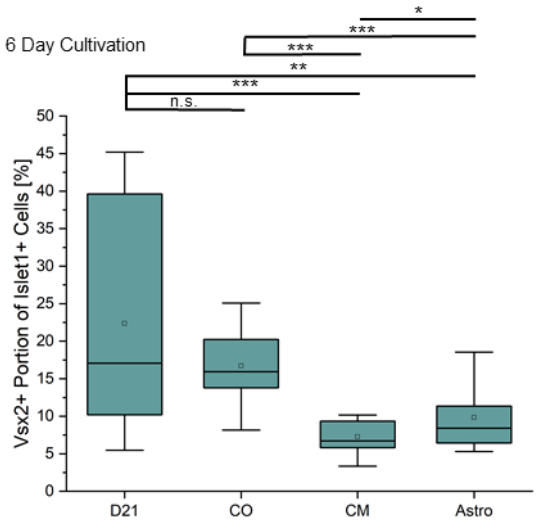
Supplementary Figure 1: Micrograph of glia specific marker proteins in the murine retina and long term cultivated ROs. *Cross-section through the retina of a p28 mouse showing the nuclei of MGCs stained with Sox9 and their radial processes positive for CRALBP and vimentin (**A**). ROs exhibit no signal for CRALBP after an extended cultivation until D40 or D50 (**B**).*



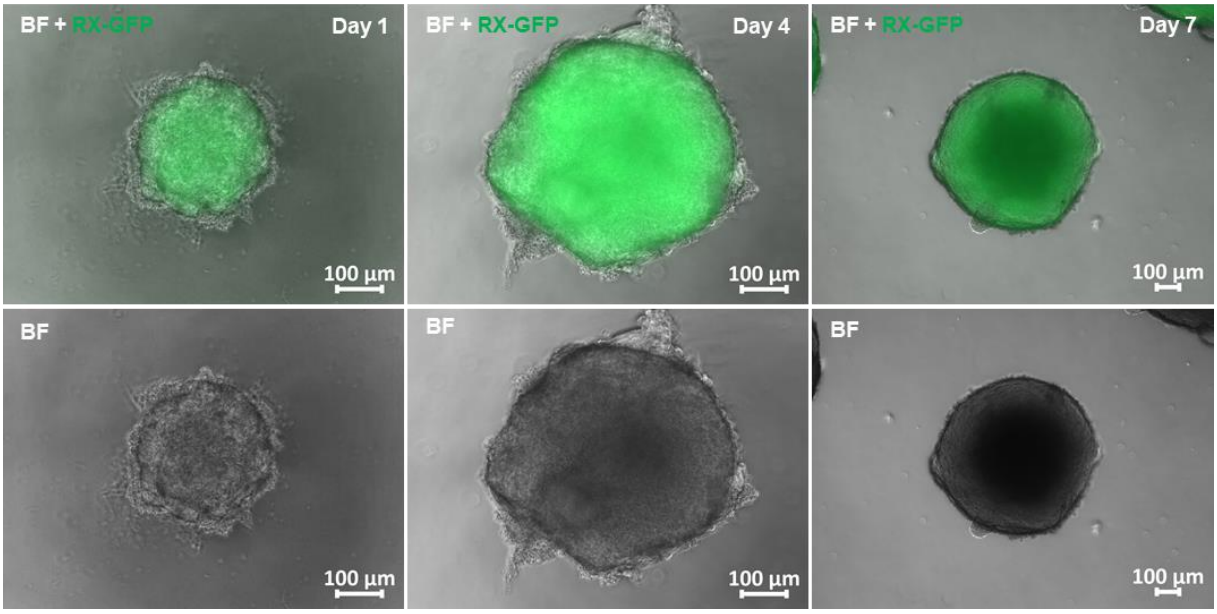
Supplementary Figure 2: Staining of glia markers in ROs of different ages. *Regular ROs display increasing numbers of cells positive for the late retinal progenitor marker NFIa starting on D21. Staining for the Müller glia specific marker CRALBP at D18, D21 or D28 showed no signal. After the treatment with the Notch ligands DLL4 and Jagged1 no signals could be detected for either NFIa or CRALBP.*



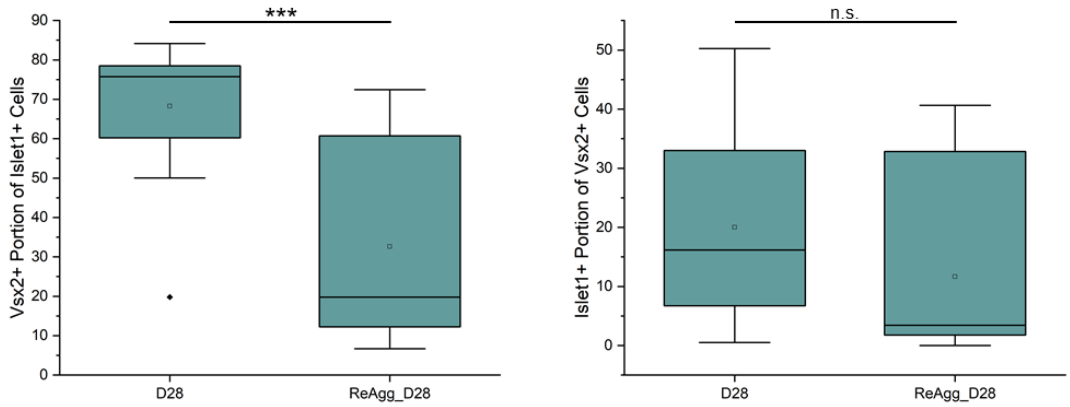
Supplementary Figure 3: Isolated primary astrocytes*. Immunostaining of the astrocyte enriched proteins Sox2 and p27kip1 in primary cortical astrocytes. Additional staining of actin displays the cytoskeleton.*



Supplementary Figure 4: *Relative cell number of Islet1⁺ cells that are also positive for Vsx2 after six days of treatment compared to untreated ROs on D21.*



Supplementary Figure 5: Growth of reaggregated ROs one, four and seven days after reaggregation. *The dissociates retinal cells form an aggregate one day after seeding and thereafter quickly grow and organise themselves. After four days the outer rim of the aggregate is smoother and first signs of a bright epithelium can be seen. Thereafter the reaggregated ROs exhibit morphology as the regular cultured ROs.*



Supplementary Figure 6*: Relative cell number of Islet1⁺ cells that are also positive for Vsx2 and vice versa in reaggregated ROs compared to untreated ROs on D28.*

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