Generation of retinal neurons

Focus on the proliferation and differentiation of the horizontal cells and their subtypes

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Abstract

We have used the chicken retina as a model for investigating cell cycle regulation and cell fate commitment during central nervous system development. This thesis focuses on the characterization of and commitment to the horizontal cell fate in the retina. Horizontal cells are interneurons that provide intraretinal signal processing prior to information relay to the brain. We have identified molecular markers that selectively distinguish the three subtypes of horizontal cells, previously described in the chicken retina based on morphology. Subtype specific birth-dating revealed that horizontal cell subtypes are generated consecutively by biased progenitors that are sensitive to the inhibitory effects of follistatin. Follistatin stimulates proliferation in progenitors by repressing the differentiation signal of activin. Initially, injection of follistatin led to a decrease in committed horizontal cells but as the inhibitory effect dissipated it resulted in an increased number of horizontal cells. During development committed horizontal cell progenitors migrate to the vitreal side of the retina where they become arrested in G2-phase for approximately two days. When the arrest is overcome the horizontal cell progenitors undergo ectopic mitosis followed by migration to their designated layer. The G2-phase arrest is not triggered or maintained by any of the classic G2-arrest pathways such as DNA damage or stress. Nevertheless, we show that the cyclin B1-Cdk1 complex has a central role in maintaining this G2-phase arrest. Two transcription factors, FoxN4 and Ptf1a, are required for the generation of horizontal cells. We show that these factors are also sufficient to promote horizontal cell fate. Overexpression of FoxN4 and Ptf1a resulted in an overproduction of horizontal- and amacrine cells at the expense of ganglion- and photoreceptor cells. We identified Atoh7, a transcription factor required for the generation of ganglion cells, as a Ptf1a transcriptional target for downregulation. Our data support a common horizontal/amacrine lineage separated from the ganglion/photoreceptor lineage by the action of Ptf1a. In conclusion, these data describe several novel characteristics of horizontal cells enhancing our understanding of neural development and cell fate commitment.

Keywords: FoxN4, Ptf1a, PH3, G2-phase, Cell cycle arrest, Differentiation, Fate, Commitment, Neurogenesis, Follistatin

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"They underestimated me"

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III  Fard, S.S., Boije, H., Hallböök, F. (2011) The terminal mitosis of chicken retinal horizontal cells is preceded by a G2-phase arrest that relies on the cyclin B1-Cdk1 complex but is independent of DNA damage. (Submitted to *The Journal of Neuroscience*)


VI  Boije, H., Fard, S.S., Ring, H., Hallböök, F. (2011) FoxN4 is sufficient for commitment to the retinal horizontal cell fate and is able to instigate differentiation programs in neural progenitors. (Manuscript)

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Cover image depicts a co-labeling of Lim1 (Red) and Isl1 (Green) in the peripheral st24 chicken retina with nuclei visualized by DAPI.
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Abbreviations

AC Amacrine Cell
Atoh1 Atonal homolog 1, a.k.a. Math1
Atoh3 Atonal homolog 3, a.k.a. Math3
Atoh7 Atonal homolog 7, a.k.a. Math5
Ascl1 Achaete-scute homolog 1, a.k.a. Mash1
bHLH basic helix-loop-helix
BP Bipolar Cell
CNS Central nervous system
E Embryonic day
FoxN4 Forkhead box N4
GABA Gamma-aminobutyric acid
GC Ganglion Cell
GCL Ganglion Cell Layer
HC Horizontal Cell
HCL Horizontal Cell Layer
INL Inner Nuclear Layer
IPL Inner Plexiform Layer
Isl1 Isl Lim homeobox 1
Lim1 Lim homeobox gene 1
MC Müller glia cell
Ngn2 Neurogenin 2
ONL Outer nuclear layer
OPL Outer Plexiform Layer
Pax6 Paired box gene 6
PE Pigment epithelium
PH3 Phosphorylated Histone 3
PR Photoreceptor
Prox1 Prospero homeobox protein 1
Ptf1a Pancreas transcription factor 1 alpha
RPC Retinal Progenitor Cell
Shh Sonic hedgehog
St Stage (according to Hamburger and Hamilton, 1951)
TF Transcription factor
TGF-β Transforming growth factor beta
Vsx2 Visual system homeobox 2, a.k.a. Chx10
Chapter I - Introduction

Formation of the central nervous system, the general issue at hand

The human brain is estimated to contain 100 billion neurons of roughly 10,000 different cell types each making 1 to 100,000 synaptic connections to other cells. In order to understand how this intricate organ functions, with its extreme structural and functional diversity, we need further knowledge of how it is formed. During the formation of the central nervous system (CNS) cells are faced with some fundamental decisions; to proliferate and expand the progenitor pool or to exit the cell cycle and differentiate into neurons or glia. This process needs to be under strict control since premature cell cycle exit would deplete the progenitor pool and lack of neurogenesis would leave the CNS missing differentiated neurons. Both these events would rule out the generation of functional circuits. Identifying intrinsic and extrinsic factors responsible for cell cycle control and fate commitment is crucial for our understanding of the development and function of the CNS.

The retina, with its relative simplicity, has been a popular model for studying the development of the CNS. The field of retina research was born in the late nineteenth century when Ramón y Cajal, in 1893, utilized Golgi’s ‘new’ silver staining technique to provide the first anatomical organization of the vertebrate retina (Ramón y Cajal, 1972). Analysis and manipulations is facilitated by the well-characterized cellular architecture and its accessibility. Another advantage is that the eye, which is a non vital organ, allow for conditional genetic manipulations that would otherwise be embryonic lethal. The chicken retina was used during the work of this thesis in an effort to unravel how neurons are generated during development.

An introduction to the eye

Origin, anatomy and function of the retina

During gastrulation eye development is initiated as a single field located centrally in the developing forebrain. This eye field was initially identified
by transplantation and fate-mapping experiments (Adelmann, 1929a and b). Currently, the presence of a set of transcription factors (TF) is used to define the eye field. Genes involved in specifying the eye field are highly conserved through evolution and include Otx2, Six3, Rx, Tbx3, Pax6, Lhx2 and Six6 (reviewed in Levine and Green, 2004). Loss of Rx, Six6 and Pax6 results in different grades of eye developmental defects (Zhang et al., 2000). Overexpression of Pax6, Six3, Rx and Six6 can expand or induce eye tissue in the vertebrate nervous system (Mathers et al., 1997; Chow et al., 1999; Loosli et al., 1999). Pax6 has for a long time been crowned the master control gene in eye development (Gehring, 1996). Overexpression of Pax6 results in the formation of ectopic eyes while homozygous mutants have only remnants of ocular tissues (Chow et al., 1999; Hill et al., 1991). However, conditional knock-out mutants for Pax6 have revealed that, although Pax6 is essential for the formation of the lens placode and the lens, it is not required for the formation of the optic cup (Lang, 2004).

The eye field is separated into two regions during the establishment of the midline. The diencephalon bulge out and form the optic vesicles that terminate close to the surface ectoderm (Fig. 1A). Thickening of the surface ectoderm forms the lens placode that interacts with the optic vesicle and stimulate an inward fold creating the bilayered optic cup (Fig. 1B). The inner layer of the optic cup gives rise to the neural retina, whereas the outer layer will form the retinal pigment epithelium (Fig. 1C). The optic vesicle remains connected to the forebrain by the optic stalk, which will later give rise to the optic nerve. The central part of the optic cup is populated by multipotent retinal progenitor cells (RPC) that will differentiate into five types of neurons, ganglion cells (GCs), photoreceptor cells (PRs), horizontal cells (HCs), amacrine cells (ACs) and bipolar cells (BPs), and one type of glia cell, the Müller cell (MC) (reviewed in Masland, 2001; Marquardt and Gruss, 2002).

Figure 1: Schematic visualization of the early developing chicken eye. ov, optic vesicle; os, optic stalk; nr, neural retina; pe, pigment epithelium; le, lens; st, stages according to Hamburger and Hamilton, 1951.

The mature retina is a highly structured tissue with three nuclear layers interspersed by two plexiform layers containing the majority of synaptic connections (Fig. 2). The outer nuclear layer (ONL) contains the nuclei of the cone and rod PRs responsible for the conversion of incoming photons into a
change in membrane potential. Rods are mainly involved in vision under dim conditions while cones are responsible for color vision and the vision in normal lighting. The inner nuclear layer (INL) harbors the HCs, BPs and ACs. The PRs connect to the BPs in the outer plexiform layer (OPL) in order to relay the signal while the HCs utilize inhibitory synapses on both to modify the output signal. The nuclei of the MCs are also situated in the INL but their somas span the entire thickness of the retina, providing structural and homeostatic support. MCs buffer potassium and facilitate the removal of GABA and glutamate from the synaptic areas. The ganglion cell layer (GCL) is composed of GCs and displaced ACs. In the inner plexiform layer (IPL) the BPs relay the information to GCs. The ACs modulate the signal from the BPs in the IPL by providing inhibition directly to the GCs and by modulating transmitter release from the BPs. The axons of the GCs bundle up and form the optic nerve that in humans partly projects to the lateral geniculate nucleus with secondary contacts to the primary visual cortex in the brain. There are several other targets in the human brain involved in balance, regulation of circadian rhythms and reflex control of pupil and lens.

![Diagram of neuronal layers in the retina](image)

**Figure 2:** Schematic representation of the neurons in the vertebrate retina. PR, photoreceptor; HC, horizontal cell; BP, bipolar cell; AC, amacrine cell; GC, ganglion cell; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

**Temporal and spatial neurogenesis in the retina**

The different types of cells in the retina are born during distinct but overlapping time-windows throughout development and this order is highly conserved between species. Their birth can roughly be divided into an early phase, where GCs, cone PRs and HCs are formed, and a late phase, where
BPs, rod PRs and MCs are generated (Mey and Thanos, 2000). The ACs are born in between these phases, overlapping with both. In chicken, the majority of the retinal cells are born between embryonic day 3 (E3) and embryonic day 9 (E9) in the following order; GCs, HCs, cone PRs, ACs, BPs, rod PRs and MCs (Fig. 3) (Prada et al., 1991). In the rat, the GABAergic ACs are born first among the ACs, while acetylcholine and dopamine expressing ACs are not generated until two to three days later (Lee et al., 1999). This indicates that the different subtypes are not generated during the maturation process but rather born into a specific subtype fate. The extended period of HC development shows that there may be a long interval from the birth of a cell to its final differentiation. HCs are among the first neurons to be generated; however Zimmerman et al. (1988) found that the HCs in the cat retina arrived to their designated layer three weeks prior to differentiation.

Differentiation is initiated in the central part of the retina and progresses towards the periphery in a wave-like fashion resulting in a more mature retina in the central part than in the peripheral part (Prada et al., 1991). Studies in the chicken retina suggest a role for Shh and fibroblast growth factor (FGF) signaling in the spread of this neurogenic wave (Zhang and Yang, 2001a, b; McCabe et al., 1999). Blocking the FGF receptor retards the front of GC genesis whereas application of exogenous FGF1 caused generation of GCs in the peripheral retina (McCabe et al., 1999). Mutations in Shh or blocking of Hedgehog signaling impede the spread of the GC wave front (Neumann and Nuesslein-Volhard, 2000). Differentiated GCs secrete Shh which is required to promote the progression of the differentiation wave front and to negatively regulate the GC genesis behind the wave front (Zhang and Yang, 2001b).
Cell cycle progression in retinal cells

During cell cycle progression in the retina a G1-phase cell enters the S-phase and DNA synthesis occurs whilst the nucleus is migrating towards the vitreal side (Fig. 4A). During the migration cells are attached to both the ventricular and the vitreal side with the nucleus undergoing interkinetic nuclear migration (INM). The neuroepithelium spanning the migratory pathway forms the ventricular zone. In the replicated state G2 the nucleus migrates back to the ventricular surface where mitosis occurs (Fig 4A). The daughter cells either exit from the cell cycle and differentiate, or re-enters into the cell cycle and initiates DNA synthesis once more (Fig. 4A, B). The length of the cell cycle varies during development. Chicken RPCs complete a cell cycle in 5 hours at st7 whereas it takes 10 hours in a st29 embryo (Fujita, 1962). Other animals, exemplified by rat, show an even greater lengthening of the cell cycle; 14h at E14, 36h at P1 and 55h at P8 (Alexiades and Cepko, 1996). These fast cell cycles during early development are crucial in scaling the output of progenitors in the optic vesicle and cup to compensate for the increase in the size of the retina. During early retina development cell divisions are symmetrical, giving rise to two daughter cells that continue cycling to expand the progenitor pool (Fig. 4A). At later stages, the RPCs are more prone to divide asymmetrically, giving rise to one daughter cell that return to the cell cycle while the other one exit the cell cycle and differentiate (Fig. 4B). At even later stages there are symmetrical divisions giving rise to two post-mitotic daughter cells (Fig. 4C).

![Figure 4: Cell cycle progression during retina development.](image)

To cycle or not to cycle - Proliferation versus differentiation

If too many cells exit the cell cycle there will be an increase in the proportion of early born cell types with a depletion of the RPC pool and a resulting
decrease in later born cells. Controlling asymmetry in the RPC pool and thereby ensuring the continued production of progenitors requires both extrinsic signaling and intrinsic inhibition.

Notch and Delta act as receptor and ligand in the RPCs and regulate proliferation and differentiation through lateral inhibition. A cell responds to inductive cues in the environment and through a feedback loop it prevents its neighbors from doing the same. Notch activation inhibits the expression of Delta and the cells receiving the lateral inhibition lose the ability to signal to their neighbors. This prevents all RPCs from leaving the cell cycle in response to inductive cues thus ensuring continued proliferation. An important aspect of lateral inhibition is that cells are initially equipotent. Studies revealed that misexpression of an activated form of Notch kept progenitors in an undifferentiated state while expression of a dominant-negative Delta protein caused the RPCs to adopt the early fates generated in the retina; cone PRs and GCs (Rapaport and Dorsky, 1998; Austin et al., 1995). When the dominant-negative Delta protein was expressed at later stages of development it resulted in an increase in later generated cell types, primarily rod and cone PRs at the expense of MCs and BPs (Dorsky et al., 1997). Two downstream targets of the Notch signaling pathway, bHLH TFs Hes1 and Hes5, regulate proliferation in the retinal precursor cells (Ohsava et al., 2008; Ohtsuka et al., 1999). Misexpression of Hes1 in the retina inhibited neuronal differentiation and maintained progenitors whereas Hes1-null mice showed reduced proliferation and premature differentiation (Tomita et al., 1996; Kageyama et al., 1997). In the absence of Hes1, Atoh1 expression was upregulated which accelerated neuronal differentiation that resulted in an increase of early-born neurons at the expense of late-born types (Ishibashi et al., 1995).

Receiving the go-ahead signal to exit the cell cycle in response to an inductive cue is however not sufficient. Cycling kinase inhibitors are factors that prevent cells from re-entering the cell cycle after their terminal mitosis. At least two of these inhibitors, p27Kip1 and p57Kip2, are expressed in mutually exclusive populations of RPCs (Dyer and Cepko, 2000). Overexpression of p27Kip1 and p57Kip2 led to premature cell cycle exit through interactions with Cyclin D1 (Dyer and Cepko, 2001). Mice lacking p27Kip1 or p57Kip2 exhibited inappropriate S-phase entry and increased apoptosis (Dyer and Cepko, 2001).

In order for a progenitor cell to exit the cell cycle and differentiate it has to translate extrinsic signaling to intrinsic inhibition of proliferative signals and follow through by preventing cell cycle re-entry.
The competence model - a hypothesis on retinogenesis

There are two important observations that form the current view on retina development; first, the highly conserved order in which the retinal cell types are generated in mammals, birds, and fish. This is despite the large difference in the duration of retinogenesis in between species from several weeks to just a few days. Second, the lack of lineage restriction; all retinal cell types can be generated from a common precursor (Wets and Fraser, 1988; Turner and Cepko, 1987; Turner et al., 1990). These observations combined with some crucial experiments, gave the fundament to the competence model in which RPCs are thought to undergo a series of competence stages from which different cell types are generated during development. When a RPC leave the cell cycle, intrinsic factors convey the competence to generate a certain cell type. Over time this competence change and other types of neurons are generated. When the intrinsic competence changes it may affect the expression of cell surface receptors allowing a different response to the same extrinsic signal. Experiments revealed that RPCs from different developmental stages respond differently to mitogens and that their levels of epidermal growth factor receptors change over time (Lillien and Cepko, 1992). When explanted into a developing retina of another age, chicken retinal progenitors that would normally generate GCs continued to do so regardless of the age of the environment they were placed in (Austin et al., 1995). While environmental factors can influence the relative proportions of different cell types produced, they are not able to generate temporally inappropriate cell types from RPCs (Belliveau et al., 2000). RPCs during late retina development are not able to generate the cell types normally generated early and early RPCs do not have the competence to generate cell types normally formed late during development.

Previously, postmitotic daughter cells were thought to be uncommitted as they left the cell cycle and migrated to a particular layer and adopted its fate. This was however contradicted by the expression of cell-specific markers directly after completion of the M-phase (Waid and McLoon, 1995). A study performed by Cayouette and colleagues (2003) plays down the significance of extrinsic cues since they found that dissociated rat retinal cells as early as E16 were fate-determined and only driven by intrinsic cues. Progeny of the same proportions and clonal compositions were observed in the dissociated cells as progenitors in vivo (Cayouette et al., 2003). Moody et al. (2000) showed by transplantation of individual Xenopus blastomeres that these were at an early stage intrinsically biased to produce neuropeptide Y and dopamine-positive amacrine cells. The competence state seems to be intrinsically determined whereas a specific cell fate within a certain competence state might be regulated to a large degree by extrinsic signaling (Fig. 5).
Temporal regulation of progenitor competence

Two genes have been shown to confer temporal competence of RPCs, Ikaros and growth differentiation factor 11 (Gdf11) (Elliott et al., 2008, Kim et al., 2005). The presence or absence of Ikaros and Gdf11 alter RPC competence thereby altering the cell types generated. Ikaros is the mouse ortologue of Hunchback (Hb), a Drosophila zinc finger TF known to specify early-born neuronal identity in the Drosophila neuroblast lineage (Novotny et al., 2002). During retina development Ikaros is both necessary and sufficient to confer early neural type competence to mouse RPCs. Ikaros is expressed by early but not by late RPCs and its misexpression was sufficient to confer competence to generate early born neurons in postnatal RPCs (Elliott et al., 2008). Loss of function mutants of Ikaros showed a decrease in early generated neurons whereas late born neurons were not affected.

Gdf11 controls RPC competence within the early generated cells and affects the number of GCs, ACs and PRs generated. Gdf11 does not affect proliferation but its expression negatively regulates GC genesis in mice. Gdf11 treated explants showed a large reduction in the number of GCs. Gdf11 null mutant mice had 50% more fully differentiated GCs at E17.5 likely due to a lengthening of the period where GCs could be generated (Kim et al., 2005). Follistatin (Fst), a Gdf11 sequestering molecule, is also expressed during early retinogenesis and loss of this inhibition in the Fst-/- retina shortened the window of GC genesis and caused a reduction of GCs. Manipulating Gdf11 expression effected GC, PRs and ACs but not HCs, suggesting heterogeneity in the RPC pool with respect to Gdf11 response (Kim et al., 2005). In the spinal cord Gdf11 is expressed by newly born neurons, but not in progenitor cells, regulating the proliferation and differentiation of surrounding cells (Shi and Liu, 2011). Gdf11-/- animals displayed higher proliferation and less differentiation suggesting that cells remained as...
progenitors. Shi and Liu demonstrated that Gdf11 facilitates cell cycle exit and neuronal differentiation through its ability to upregulate p57Kip2 and p27Kip1 while downregulating Pax6 expression.

The existence of Ikaros supports the competence model but the Gdf11 experiments reveal both that there are finer subdivisions of competence and that cells within a competence phase are not a homogeneous group.

Fate commitment

Heterogeneity in the progenitor pool

One important question is whether the RPCs can be viewed as a homogeneous or heterogeneous pool during development. As discussed previously in the sections on cell cycle regulation and temporal competence; RPCs at a given time during development utilize different factors to ensure their cell cycle exit, and over a temporal aspect, the progenitor pool alters competence. Affymetrix analyses on single RPCs revealed extensive heterogeneity. Although most RPCs expressed Pax6, Vsx2 and Sox2 there was variation in other factors and a clear difference between early and late RPCs (Trimarchi et al., 2008). This implies that there is heterogeneity in the RPC pool.

Monoclonal antibodies against VC1.1 and Syntaxin both label HCs and AC in the P8 rat retina (Alexiades and Cepko, 1997). These epitopes were also identified on a subpopulation of RPCs and the post-mitotic daughters of these progenitors were biased to adopt HC or AC fate (Alexiades and Cepko, 1997). Simultaneously VC1.1 negative progenitors generated primarily cone PRs. This clearly identifies heterogeneity among the RPCs but this heterogeneity reflects a predisposition for rather than a commitment to a specific fate.

Cyclin D1 is expressed in RPCs and the null mouse has hypocellular retinas due to reduced proliferation and increased PR apoptosis postnatally (Das el al., 2009). Cyclin D1 promotes progression from G1 to S-phase by activating cyclin dependent kinases 4 or 6 (CDK4/6) and by sequestering cyclin dependent kinase inhibitors such as p27Kip1 (Kozar and Sicinski, 2005). Consistent with the increased frequency of cell cycle exits in neurons lacking Cyclin D1 there was an increase in the number of early born GCs and PRs but the onset of neurogenesis was not altered. Interestingly, the number of HCs and ACs, the other cell types generated early, decreased. There was a significant reduction in the number of cells labeled for Ptf1a, a bHLH TF which labels progenitors biased to the HC and AC fate. Inactivation of p27Kip1 restored the balance in the RPC pool to a more normal cell type distribution. The kinase inhibitors, p27Kip1 and p57Kip2, are expressed in mutually exclusive populations of RPCs demonstrating heterogeneity in the RPC
pool with respect to their expression of cell cycle regulators (Dyer and Cepko, 2000). Expression of p57<sup>Kip2</sup> was observed between E14.5 and 16.5 in approximately 16% of the RPCs exiting the cell cycle during this time (Dyer and Cepko, 2001). The temporal expression of p57<sup>Kip2</sup> coincides with HC genesis and the p57<sup>Kip2</sup>-/- mice revealed a subpopulation specific defect among the ACs (Dyer and Cepko, 2000). Combining these results allows for speculation that HCs and ACs utilize p57<sup>Kip2</sup> during cell cycle exit while GCs and PRs use p27<sup>Kip1</sup>.

There are several reports suggesting that HCs and ACs share a common biased progenitor. Either the mode of cell cycle exit affect the fate of cells or heterogeneity in the RPC pool is reflected in their use of cyclin kinase inhibitor, either way it suggests a link between cell cycle exit and cell fate determination.

**Fate commitment in retinal neurons**

There is an abundance of intrinsic and extrinsic factors involved in specifying cell fate in the retina (Harris, 1997; Levine and Green, 2004; Yang, 2004; and references therein). Neurotrophic factors, growth factors, retinoic acid and Shh are a few of the extrinsic effectors active in retinogenesis. However, these secreted factors are interpreted by receptors that are primarily under intrinsic regulation of TFs. If TFs regulate the expression of the receptors responsible for sensing the extrinsic signals it allows one to view fate commitment as an intrinsic affair. It is not the scope of this segment to give a comprehensive account of all TFs known to influence particular cell fates from the RPC pool. Particular examples will be made to introduce the complexity of the system during fate commitment. Genes involved in the generation of the HCs will be dealt with in the next chapter.

TFs from basic helix-loop-helix (bHLH) and homeodomain families are important during retina development. There are five known retinal neuron-promoting bHLH genes; Atoh7, Ngn2, Atoh3, NeuroD1 and Ascl1 (Brown et al., 1998). These are activated in sequence in the mouse retina with Atoh7 first, followed by Ngn2 and NeuroD1. Atoh7 is essential for GC differentiation; however, lineage tracing revealed that Atoh7 expressing cells also gave rise to HCs, ACs and some PRs (Yang et al., 2003). Atoh7 normally inhibits NeuroD1 and Ngn2, two genes known to promote PR fate. In Atoh7-/- mice RPCs are unable to adopt GC fate and the released block of Ngn2 and NeuroD1 resulted in an increase of cones PRs (Le et al., 2006). Not all cells expressing Atoh7 become GCs and overexpression of Atoh7 showed that GC fate was promoted in approximately half of the affected cells (Kanekar et al., 1997). Negative feedback from GCs, by secreted factors, has been shown to limit the generation of new GCs (Waid and McLoon, 1998). Furthermore, selective depletion of GCs leads to an increase in the number of progenitors...
expressing Atoh7 providing a link between extrinsic and intrinsic coordination of cell division and cell fate (Mu et al., 2005).

Although bHLH TFs can promote neurogenesis they can not correctly specify a specific neuronal cell type without a homeodomain TF (reviewed by Hatakeyama and Kageyama, 2004). One example is the generation of BPs. BPs requires bHLH TFs Ascl1 and Atoh3 for their formation, but their misexpression predominantly generates PRs. The homeodomain TF Vsx2 is also required for BP development but its overexpression induces MCs and undifferentiated cells situated in the INL. However, combining the misexpression of Vsx2 with either Ascl1 or Atoh3 significantly promoted the generation of BPs (Hatakeyama et al., 2001). It is suggested that homeodomain TFs specify the layer identity and that bHLH TFs participate in cell type specification.

These examples provide an initial glance of the complex mesh of multiple intrinsic factors that coordinate the formation of the retinal neurons.

Brief summary of Chapter I

The eye is specified early during development by a set of TFs in the anterior neural tube. Consecutive evagination and invagination creates a bilayered optic cup where the inner layer will give rise to the neural retina. RPCs in the optic cup are multipotent and will give rise to the five types of neurons found in the mature retina. Cycling progenitors undergo interkinetic nuclear migration, with mitoses occurring at the ventricular surface. Cell cycle exit is under both extrinsic and intrinsic control. The different cell types in the retina are generated in a temporal conserved order in a central to peripheral manner. The competence model hypothesize that RPCs undergo a series of intrinsic competence stages altering the type of cell born in response to extrinsic signaling. TFs are pivotal in commitment to a specific cell type but several TFs from different families need to collaborate in order to promote a particular fate.
Chapter II - Our studies

"People who count their chickens before they are hatched act very wisely, because chickens run about so absurdly that it is impossible to count them accurately."

Oscar Wilde

During our inquiry into fate commitment and differentiation in the retina we have focused on the horizontal cells (HCs). There are several reasons why the HCs are of particular interest. They have clearly distinguishable subtypes, both by morphological criteria and by molecular markers, allowing study of subtype formation. The number of HCs is not adjusted by apoptosis, suggesting that the number of cell divisions is under strict control. They undergo bi-directional migration, resulting in a stopover on the vitreal side before arriving at their destined layer (Edqvist and Hallböök, 2004). Recent studies in zebrafish suggest that HCs undergo ectopic mitoses in the horizontal cell layer, and that mature HCs can be forced to re-enter the cell cycle and form retinoblastoma in a murine model (Godinho et al., 2007; Ajioke et al., 2007). The work of this thesis can be divided into two parts; Papers I-III deal with the characterization of the HC subtypes, their birth and migration, whereas papers IV-VI are focused on the commitment to the HC fate.

Specific aims of the thesis

The aim of Paper I was to molecularly characterize the three HC subtypes, which have previously been described morphologically. We also wanted to birth-date the different subpopulations to determine whether they were generated simultaneously or consecutively. Previous studies had reported ectopically located HCs following prolonged follistatin exposure, a factor that neutralizes members of the TGF-beta super-family (Moreira and Adler, 2006). We wished to further investigate the effect of follistatin on the generation of HCs. Paper II focused on the bi-directional migration of HCs. The increase of HCs during their migration prompted us to investigate the mitotic pattern of chick HCs. In Paper III we wanted to further characterize the HC
specific G2-phase arrest and ectopic mitoses that were discovered during the work of Paper II.

The aim of Paper IV was to study the temporal and spatial expression patterns of FoxN4 and Ptf1a in relation to HC markers Prox1, Lim1 and Isl1. FoxN4 and Ptf1a knock-out mice have recently shown that these TFs are required for commitment to the HC fate (Li et al., 2004; Fujitani et al., 2006). In Paper V and VI the aim was to investigate if Ptf1a and FoxN4 were also sufficient for commitment to HC fate. We were also interested in whether Ptf1a and FoxN4 would be sufficient for HC genesis in general or if they would promote a specific subtype of HCs.

An introduction to horizontal cells

HCs are interneurons that work as modulators of signals contributing to intraretinal signal processing before the information is relayed to the brain. The HCs have dendrites connecting to both PRs and BPs and they modulate the signals by means of inhibitory synapses primarily using GABA and glycine. HCs are among other things involved in enhancing contrast between light and dark regions and are found in all studied vertebrate retinas. Most species have two types of HCs, the axon-bearing and the axon-less (Gallego, 1986; Peichl et al., 1998). The axon extends parallel to the OPL for some distance before generating a dendritic tree. The nocturnal rodents, including mice, only have the axon-bearing HCs. The axon-less HCs, which mainly connect to cones, were likely lost during the transition to a nearly cone-free retinas, an adaption to their nocturnal lifestyle. However, in several other species, including zebrafish, turtle, pigeon and rabbit, two axon-less types of HCs have been identified (Connaughton et al., 2004; Cuenca et al., 2000; Mariani, 1987; Lyser et al., 1994). The different axon-less HCs show some bias towards which type of cones they make synapses with (Gallego, 1986).

Subtypes in the chicken retina

The chicken HCs form a distinct layer of cells, commonly referred to as the horizontal cell layer (HCL), in the outermost part of the INL. There are three different subtypes of HCs in the chicken retina based on morphology and molecular markers (Genis-Galves et al. 1979, Paper I). The H1 subtype is axon-bearing and has a ‘brush-shaped’ morphology (Fig. 6A). The H2 and H3 subtypes are axon-less and have ‘stellate’ and ‘candelabrum-shaped’ morphology respectively. The brush-shaped HC axon-terminus mainly connects to rod photoreceptors, whereas the dendritic trees of all three HCs primarily form connections with cone PRs (Zhang et al., 2006a and b). This morphological classification can also be accomplished by molecular markers (Paper I). All HCs express Prox1 and Pax6 whereas Lim1 and Isl1 are each
expressed in half the HCs in a non-overlapping manner (Fig. 6B, Paper I). The axon-bearing HCs express Lim1 while the axon-less subtypes, H2 and H3, express Isl1 (Fig. 6C, Paper I). The H2 population can be defined by Isl1 and GABA expression whereas the H3 population expresses Isl1 and TrkA (Fig. 6C, Paper I). Prox1 and Pax6 are homeodomain TFs. Lim1 and Isl1 belong to the LIM/homeodomain family of TFs, which contain a LIM domain, a unique cysteine-rich zinc-binding domain, as well as a homeodomain. GABA is the chief inhibitory neurotransmitter in the CNS. TrkA is a member of the neurotrophic tyrosine kinase receptor (NTKR) family and calretinin is an intracellular calcium-binding protein. Prox1 and Isl1 are expressed by several other cell types in the retina but the combination of Prox1 and Isl1 is exclusive to the HCs (Paper I).

Figure 6: The morphological and molecular classification of the three subtypes of horizontal cells in the chicken retina (Paper I). (A) The brush-shaped HCs express Lim1 whereas the stellate and candelabrum subtypes can be defined on their expression of Isl1 and GABA or TrkA. (B) No overlap is observed between Lim1 and Isl1 in a flatmounted P0 retina. (C) Prox1 and Pax6 are expressed in all HCs whereas the Lim1 and Isl1 split the population in two equal proportions. at, axon terminus.

The birth of a horizontal cell

S-phase and migration

Subtype specific birth-dating of the HCs by incorporation of \(^{3}\text{H}\)-Thymidine revealed a clear temporal difference as the Lim1 population was generated approximately one day prior to the Isl1 positive HCs (Fig. 7A, Paper I). The majority of the Lim1 and Isl1 populations underwent their final S-phase between E3-4 and E4-5 respectively. As previously discussed; the proposed model sees multipotent progenitors which undergo their final mitosis at the
ventricular surface followed by migration to their destined laminas. Instead of migrating directly from the ventricular surface to the HCL, the chicken HCs migrates bi-directionally to the GCL before ending up in the HCL (Fig. 7B) (Edqvist and Hallböök, 2004). The Lim1 expressing HCs not only underwent their S-phase one day earlier than the Isl1 population but also finished their retrograde migration to the HCL one day earlier (Fig. 7C-L, Paper I).

Conditional Lim1 mutant mice showed that ablation of Lim1 caused ectopic HCs with a morphology resembling that of ACs (Poché et al., 2007). Expression of Ptf1a, Prox1 and other HC markers was initiated as in wild type mice but the retrograde migration was inhibited. The ectopic HCs adopted AC morphology but did not express AC, BP or GC specific markers suggesting that proper fate commitment had occurred but that they had an erroneous laminar position (Poché et al., 2007). This indicates that Lim1 is a crucial homeodomain TF for the positioning of HCs in the mouse retina.

Figure 7: Birth-dating and migration pattern of horizontal cells (Paper I). (A) Fractions of HCs positive for [³H]-dT incorporation combined with either Prox1 (light grey bars) or Lim1 (dark grey bars) analyzed at st35. The line indicates the difference between the Prox1 and Lim1 [³H]-dT-labeled fractions and represents the birth curve for Isl1 HCs. Solid portion of the line corresponds to significant differences. (B) Schematic visualization of the HC migration with the approximate stages for the Lim1 population. (C-L) The Lim1 population migrates from the vitreal side approximately one day prior to the Isl1 population (defined as cells double positive for Prox1 and Isl1). (*) marks where the majority of cells are migrating and (**) where they have reached the HCL. Arrows in (H) indicate double labeled cells. E, embryonic day; inl, inner nuclear layer; gcl, ganglion cell layer; st, stage.
G2-phase arrest and ectopic mitosis

The function and cause of the bi-directional migration of the HCs was, and to some part still remains, an unanswered question. The majority of Lim1 positive HCs are generated between E3 and E4, but do not reach the HCL until E8, revealing a time window of unknown function. During the stopover at the vitreal side we observed that the number of Lim1 positive HCs increased. It was recently shown by Godinho et al. (2007) that HC precursors in zebrafish underwent symmetric divisions in the HCL. Godinho and colleagues speculated that HC-committed progenitors exist early during neurogenesis with a delayed terminal mitosis. This type of committed HC-progenitor has also been suggested to exist in the chicken retina (Rompani and Cepko, 2008). It has also been reported that cells with differentiated HC features can divide in a murine model of retinoblastoma (Ajioke et al., 2007). In light of this we analyzed the mitotic pattern of cells expressing HC markers. We showed that there are indeed committed HC progenitors in the developing chicken retina and that they undergo mitoses ectopically at the vitreal side (Fig. 8, Paper II). Following the temporal pattern of S-phase for the different HC subtypes the Lim1 population executed their ectopic mitoses one day prior to the Isl1 HCs (Fig. 8B). The progression of the ectopic mitoses occurred in a central to peripheral fashion that followed the maturation of the retina. We further demonstrated that all vitreal mitoses were performed by HC-committed progenitors (Paper II). The second to last division of the HC progenitors, which occurs at the ventricular side, was followed by immediate entry into S-phase during their migration to the vitreal side (Paper II). Upon reaching the GCL the HCs were arrested in G2-phase for approximately two days prior to their ectopic mitosis promptly followed by their retrograde migration (Paper II).
The length of the G2-phase arrest was not fixed. This was demonstrated by labeling the last portion of Lim1 HCs in their S-phase followed by analysis of ectopic mitoses at various time points (Paper III). S-phase labeled cells were found to undergo mitosis rather evenly distributed during the observed time-points. The variable length of G2-phase was believed to be due to regulation within the individual HC rather than to a coordinated extrinsic signal.

Two main pathways are responsible for G2-phase arrest. One pathway prevents cells from entering M-phase when DNA is damaged or not fully replicated and this path is mediated by ataxia telangiectasia mutated (ATM) and Rad-3 related (ATR). The other pathway prevents M-phase entry in cells that are stressed and this response is based on the stress induced p38
mitogen-activated protein kinase (p38MAPK). Our results indicate that the G2-phase arrest observed in HCs during the development of the chick retina is not triggered or maintained by any of the classical G2-phase arrest pathways (Paper III). Although the initial cause of the G2-phase arrest has not yet been identified, manipulations of the cyclin B1/Cdk1 complex show that nuclear localization and activation of this complex is required for progression into M-phase. Forced accumulation of cyclin B1 in the nucleus caused a premature entry into M-phase while inhibition of cyclin B1/Cdk1 activation completely blocked HC mitosis (Fig. 9, Paper III).

Figure 9: The HCs require activated cyclin B1/Cdk1 to overcome the G1-phase arrest (Paper III). (A) Forced accumulation of cyclin B1 in the nucleus by Leptomycin B caused a premature entry into M-phase. (B) Inhibition of cyclin B1/Cdk1 activation by Cdk1 inhibitor III completely blocked HC mitosis. (C) Schematic figure of the pathways regulating G2/M transition. Inhibition of ATM/ATR and Chk1 did not affect HC mitoses.
Commitment to the horizontal cell fate

There are several TFs that have been associated with HC genesis. The bHLH genes NeuroD1 or Atoh3 combined with either of the two homeodomain genes Pax6 or Six3 result in an increase of ACs and HCs (Inoue et al., 2002). The combination of Atoh3 with Pax6 seems to generate HCs more preferentially than ACs (Inoue et al., 2002). Triple knock-out mice lost their HCs in the Ngn2, Atoh3 and NeuroD1 mutant and HCs were decreased in the Ascl1, Ngn2 and Atoh3 mutant. However, none of the double mutants Ascl1, Ngn2; Atoh3, Ngn2 and NeuroD1, Ngn2 showed any defect in HC genesis (Akagi et al., 2004). This suggests that there is a wide range of bHLH genes involved during HC genesis. An ambitious systematic approach to co-overexpression of combinations of six bHLH (NeuroD1, Ngnr-1, Atoh3, Atoh7, Ascl1, Ascl3) with eight homeodomain (Rx1, Six6, Six3, Pax6, Otx2, Otx5b, BH, Vsr2) TFs in the Xenopus retina revealed several interesting combinatorial effects. The preference for HC genesis when combining Atoh3 and Pax6 was confirmed but the combination of Ascl3 with Six6 was also found to result in more HCs (Wang et al., 2005). Two other mutants, Ptf1a-/- and FoxN4-/-, revealed very specific phenotypes with a complete loss of HCs and a decrease in AC number (Li et al., 2004; Fujitani et al., 2006). The fact that the generation of HC could be completely abolished in these mutants indicates an important role for Ptf1a and FoxN4 during HC fate commitment.

HC biased progenitors are sensitive to inhibition by follistatin

Follistatin is an endogenous inhibitor that binds and neutralizes members of the TGF-beta super-family including activin, GDF8 (myostatin) and Gdf11 (Schneyer et al., 2008). Follistatin is expressed in the developing retina and PE and its primary function is facilitated through its high affinity binding to activin, which prevents activin from signaling through its receptor (Tompson et al., 2005). Activin is a secreted mitotic inhibitor and differentiation factor expressed by cells in the prospective GCL and subpopulations of ACs in the chick retina (Belecky-Adams et al., 1999). Expression of activin has been shown to stimulate differentiation of ACs (Belecky-Adams et al., 1999). Overexpression of follistatin by RCAS retroviruses leads to a reduction of ACs accompanied by ectopic expression of Prox1 in the INL suggesting that the generation of HCs may be affected by follistatin (Moreira and Adler, 2006). In the follistatin treated eyes there was a decrease in the levels of pSmad2, a mediator of activin signaling, without changes in pSmad1/5/8, which are involved in BMP-dependent signaling (Moreira and Adler, 2006). However, experiments in the spinal cord revealed that Gdf11 also signals
through pSmad2 making it difficult to rule out its involvement in the retinal effects (Shi and Liu, 2011).

The effects of prolonged exposure to follistatin on the HCs by Moreira and Adler (2006) prompted us to undertake a more thorough analysis focused on the generation of HCs. Endogenous follistatin expression was high during the earliest phase of retinal development, however, both immunohistochemical labeling and mRNA levels dropped sharply at the time of HC generation (Fig. 10A, B, Paper I). The onset of Lim1 expression closely followed the decrease of follistatin immunoreactivity as it moved towards the periphery suggesting that low levels were required for HC genesis. Reduced levels of follistatin will likely release the block of activin signaling allowing cell cycle exit and differentiation. When exposed to a high dose of exogenous follistatin during a period crucial for HC generation both Lim1 positive, axon-bearing, and Isl1 positive, axon-less, HCs increased in number (Fig. 10C-E, Paper I). Initially the bolus injection of follistatin caused a significant reduction in the number of Lim1 positive cells (Fig. 10F, Paper I). We hypothesized that the reduction of newly generated Lim1 cells by the added follistatin was an effect on the progenitor cells generating the Lim1 positive cells. The inhibitory action of follistatin would keep the progenitor cells proliferating thus delaying the onset of Lim1 expression. This would lead to fewer newly generated Lim1 positive cells compared to controls. When the effect from the follistatin injection had dissipated a larger population of progenitors would then be allowed to instigate fate commitment programs resulting in an increased number of HCs in the mature retina. However, the follistatin-phenotype at E9 (st35) was often restricted to distinct regions of the retina. This indicates the existence of a spatio-temporal window during which HC-generating progenitor cells are susceptible to follistatin inhibition.

If a follistatin treatment early during development preferentially affected the early generated Lim1 population while a later treatment had effects on the later generated Isl1 population, it could be hypothesized that the Lim1 and Isl1 HC subtypes were generated from separate progenitor cells. Conversely, if the early follistatin treatment also had effects on the late population it is likely that both subtypes are generated from a common pool of progenitor cells that is affected by the follistatin inhibition and thus expanded. We found that both early and late follistatin treatment caused a significant increase in the total number of Prox1 positive HCs as well as significant increases in both of Lim1 and Isl1 positive HC subtypes compared to controls (Fig. 10C-E, Paper I). No effect was observed on other cell types. This suggests that there is a common progenitor giving rise to both HC subtypes. At st35, a time at which all HCs have reached the HCL in the central retina, Lim1 and Isl1, Prox1 double positive cells were observed in the INL (Figure 10G). These cells eventually reached the HCL by st36 further indicating that exogenous follistatin alters the period where HCs are generated (Paper I).
Combined, these observations indicate that follistatin stimulate proliferation in the retina, possibly by repressing activin signaling. Our interpretation is that the transient follistatin overexposure prevents HC-generating progenitor cells from producing cells committed to the HC fate. Instead, the affected progenitor cells may produce two mitotic daughters that will both inherit the capability to produce HCs. This ultimately results in an increase of the total number of HCs in the mature retina.

Figure 10: **Exogenous follistatin cause an increase in both Lim1 and Isl1 positive horizontal cells (Paper I).** (A) qRT-PCR of follistatin mRNA expression during the development of the chick retina. (B) Immunohistochemical labeling against follistatin and Lim1 at st22. (C-E) Quantification of Prox1 positive (C), Lim1, Prox1 double positive (D) and Isl1, Prox1 double positive cells (E) at st35. (F) Quantification of Lim1 positive cells 22 hours after follistatin injection. (G) Immunohistochemical labeling against Lim1, Prox1 or Isl1, Prox1 in control and follistatin injected retinas at st35.
Ptf1a drives specification of all horizontal subtypes

Ptf1a encodes a bHLH TF and was initially described as a factor driving differentiation of a pancreatic lineage (Kawaguchi et al., 2002). It has also been linked to the generation of HCs and ACs in the mouse retina (Fujitani et al., 2006). Expression of Ptf1a is lost in FoxN4-/- retina suggesting that FoxN4 is upstream of Ptf1a (Li et al., 2004). The onset of FoxN4 and Ptf1a expression in the chicken retina supports this hierarchy order during development (Paper IV). In the Ptf1a-/- mouse retina Prox1 and Lim1 expression was lost along with all HCs (Fujitani et al., 2006). Ptf1a has been suggested to determine GABAergic cell fate in the spinal cord and in the cerebellum (Glasgow et al., 2005; Hoshino et al., 2005). In contrast to the mouse retina, which only has the Lim1 expressing GABAergic HC subtype, the chicken retina also has non GABAergic HCs expressing Isl1. An important question was whether Ptf1a is involved in generating all HCs or if it is a determinant for the GABAergic fate in a subpopulation of HCs.

We found that forced expression of Ptf1a by RCAS virus lead to an increase in ACs and all HC subtypes as well as a decrease in GCs and PRs (Fig. 11, Paper V). The lamination of patches affected by Ptf1a misexpression revealed disorganized plexiform and nuclear layers. These patches also exhibited reduced proliferation and mitoses indicating that forced misexpression of Ptf1a induced premature cell cycle exit by progenitors (Paper V). Labeling for Ap2α, expressed in ACs, and Prox1 was increased both at E7 and E12 suggesting that the disrupted lamination seen at E12 is secondary to the altered fate commitment from GC/PR to AC/HC (Fig. 11A, C). Brn3a, a marker for GCs, and visinin, labeling PRs, were drastically reduced in RCAS-Ptf1a infected regions (Fig. 11A, C). These results were verified both by FACS-sorting and qRT-PCR (Fig. 11B, D). Both GABAergic Lim1 positive HCs and non-GABAergic TrkA, Isl1 double positive HCs were generated as a consequence of the Ptf1a overexpression (Paper V). This indicates that Ptf1a is involved in HC fate commitment in general rather than in GABAergic subtype specification. The 50/50 ratio between the subtypes remained unaltered in regions infected with RCAS-Ptf1a virus indicating that the subtype specificity was maintained even under these conditions (Fig. 12B, Paper V). The HCs generated were unable to migrate to their designated layer. The absence of GCs and PRs in the affected patches might cause abnormal gradients of secreted signaling molecules preventing proper migration of neurons to their designated layer. Atoh7, required for the generation of GCs, was found to be a direct transcriptional target of Ptf1a (Paper V). This suggests that the negative regulation of Atoh7 by Ptf1a is likely the cause of the fate switch from the GC/PR lineage to the AC/HC lineage.
Figure 11: Effects of Ptf1a on retinal cell differentiation (Paper V). (A) RCAS infected and RCAS-Ptf1a infected retina patches at E7 labeled against Brn3a (GCs), and gag/p27 (virus capsid protein), visinin (PRs), Ap2α (ACs and HCs) and Prox1 (HCs). (B) Cell type representation among infected cells analyzed by FACS in an E7 retina using labeling against Prox1, visinin, and Ap2α. (C) RCAS infected and RCAS-Ptf1a infected retina patches at E12 labeled against Brn3a, visinin, Prox1 and Ap2α. (D) Cell type representation among infected cells analyzed by FACS at E12 using labeling against Prox1, visinin, and Ap2α. ONL, outer nuclear layer, INL, inner nuclear layer, GCL, ganglion cell layer.

Figure 12: Ptf1a overexpression is sufficient to induce both Lim1 and Isl1 positive horizontal cell subtypes (Paper V). (A) RCAS-Ptf1a infected patches at E7 or E9 double labeled either against Lim1 and Prox1 for H1 HCs or against Isl1 and Prox1 for H2 and H3 HCs. Higher magnifications of boxed regions are shown. The vitreal side is marked for orientation purposes. (B) Quantification of Lim1 and Isl1 positive horizontal cells at E9.
FoxN4 is sufficient for commitment to the horizontal cell fate

The TF FoxN4, a member of the Forkhead box (Fox) family, has been identified as an important factor in the development of ACs and HCs in the mouse retina (Li et al., 2004). FoxN4 is expressed in the developing retina in a pattern conserved in mouse, xenopus and zebrafish (Gouge et al., 2001; Schuff et al., 2006; Danilova et al., 2004). Initially FoxN4 was ubiquitously expressed in the RPC pool in the chicken retina (Paper IV and VI). As cells leave the cell cycle and differentiate the expression became restricted to the remaining progenitor cells. The temporal and spatial expression pattern of FoxN4 in the chick retina correlates well with observations in other species at both the mRNA and protein levels. Double labeling revealed that FoxN4 was expressed in the entire early RPC population along with Pax6 and Sox2 (Paper VI). During neurogenesis stronger labeling for FoxN4 was observed in some cells. These strongly FoxN4 expressing cells had downregulated their Pax6 expression concomitantly with initiation of Lim1 expression (Fig. 13A, B, Paper VI). This suggests that low levels of FoxN4 are maintained in cycling progenitors whereas higher levels trigger commitment to the HC lineage. The ubiquitous expression of FoxN4 in RPCs raise questions as to its function since FoxN4-/- mice did not exhibit any proliferation defects. It is possible that the closely related gene FoxN2 may compensate for the loss of FoxN4. The temporal and spatial expression pattern of FoxN2 closely follows that of FoxN4 during retina development (Schuff et al., 2006).

Knock-out and overexpression studies in mice suggest that FoxN4 is both required and sufficient for the generation of ACs and that it is required for the commitment to the HC fate (Li et al., 2004). The transcription factors Atoh3, NeuroD1 and Prox1 have been proposed to be possible down-stream targets of FoxN4 (Li et al., 2004). Atoh3 and NeuroD1 are essential but not sufficient for the generation of ACs, while Prox1 is essential to promote mature HCs (Inoue et al., 2002; Dyer et al., 2003). Li and colleagues forced expression of FoxN4 at a relatively late time during mouse retina development, after the time of normal HC genesis was completed. This prompted us to investigate whether an earlier overexpression would be able to drive the production of HC in an environment with the competence to generate early born neurons. FoxN4 was indeed successful at initiating HC specific markers Prox1 and Lim1 in cells electroporated with a FoxN4-IRES-GFP construct (Fig. 13C, D, Paper VI). FoxN4 was also shown to upregulate the expression of Ptf1a, Lim1 and Prox1 at a transcriptional level (Paper V).
The lamination in the mature retina was disorganized in regions electroporated with the FoxN4 construct. Similar to the Ptf1a study, the early onset of HC markers suggest that the fate-switch occurred prior to the disruption of lamination. Numerous HCs were observed in the electroporated patches but in ectopic locations and in clusters. The defect in migration by HCs might be due to a loss of signal cues from the environment caused by the abnormal layering. However, in electroporated regions with normal lamination ectopic Lim1 positive cells were still found in the IPL (Paper VI). This indicates that there is an intrinsic cause of the migratory defect in the cells forced to adopt HC fate.

Figure 13: FoxN4 is directly involved in commitment to the HC fate (Paper VI). (A) FoxN4, Pax6 double labeling in a st24 retina. (B) FoxN4, Lim1 double labeling in a st24 retina. (C) Retina, electroporated with a FoxN4-IRES-GFP vector at st20 and collected 24 hours later, counterstained against Prox1 (C) or Lim1 (D). (E) Retina, electroporated with a FoxN4-IRES-GFP vector at st20 and collected at st35, counterstained against Lim1/Prox1 (E) or Prox1/Isl1 (F).
FoxN4 is involved in subtype identity of V2 interneurons in the spinal cord. The loss of FoxN4 eliminates the expression of the bHLH TF Ascl1 causing a fate-switch for the V2b neurons to V2a (Li et al., 2005). Overexpression of FoxN4 in spinal neural progenitors promotes the V2a fate at the expense of V2b fate. Loss of Ascl1 alone displays a similar but less severe phenotype and its overexpression causes a suppression of both the V2a and V2b fates. This indicates that FoxN4 is a decisive factor during interneuron subtype formation. Del Barrio et al. (2007) concluded that FoxN4 plays a dual role in the generation of V2a and V2b spinal interneurons. Firstly, FoxN4 initiates Notch-Delta signaling resulting in asymmetry among the progenitors required for the development of V2a and V2b interneurons. Secondly, FoxN4 plays an active role in instigating the V2b genetic program. With this in mind we investigated whether our overexpression of FoxN4 at a time when the majority of Lim1 positive HCs are generated would also affect the later born Isl1 population. Initial results indicate that the clusters of HCs formed from the electroporation consisted of both Lim1 and Isl1 HCs (Fig. 13E, F, Paper VI). This suggests that FoxN4 is not involved in HC subtype specification.
Chapter III - Conclusion

The horizontal cells and their subtypes

The HCs are generated as two distinct subpopulations, the axon-bearing and the axon-less. The axon-bearing population can be identified by the expression of Lim1 and is generated approximately one day earlier than the Isl1 expressing, axon-less, population (Paper I). Immunoreactivity to Lim1 and $^3$H-T labeling concluded that the first HCs were generated as early as st18, with a peak around st21-22. These Lim1 cells were previously thought to be post-mitotic. In reality, the migrating HCs are in fact HC-committed progenitor cells undergoing S-phase while migrating across the neural retina (Paper II). Upon reaching the prospective GCL, HCs remain in G2-phase for approximately two days before undergoing ectopic mitosis followed by migration to the HCL. The Isl1 positive HCs also undertake this migration and become arrested approximately one day later than the Lim1 positive population. The axon-less, Isl1 expressing, HCs can be further subdivided based on their expression of either GABA or TrkA. By molecular markers we were able to identify the three morphologically distinct subtypes previously described in the chicken retina (Paper I) (Genis-Galvez et al., 1979). However, in slight contrast to our findings one recent report demonstrated that in P7–P21 chicken retina a small fraction of Isl1 positive HCs expressed calretinin (Fischer et al., 2007). This observation is not supported by our P0 retinal preparations and it is therefore possible that a subset of Isl1 positive HCs turn on calretinin expression after hatching. The same study also identified what was classified as a fourth HC subtype based on analysis of HC markers. A fourth HC subtype is also described in the pigeon retina based on morphological criteria (Mariani, 1987). Our results support the division of chicken HCs into three main subtypes but the actual number and the extent of heterogeneity in HC subtypes in the chicken retina is an unsettled issue.

The atypical cell cycle

The Lim1 expression during the last ventricular mitosis suggests that these cells have initiated their differentiation process. Why the HCs re-enter the cell cycle and arrest in G2-phase for several days remains unknown. The extra cell division might be a way to expand the HC population, a phenomenon also present in the cortex (Kriegstein et al., 2006). Re-entering the cell
cycle after the differentiation process is initiated may interfere with cell cycle progression and the cell is arrested until the issue is settled. There may also be intrinsic or extrinsic signals preventing progression until the time is right. Interkinetic nuclear migration in neuronal progenitors is tightly linked with cell cycle progression. If migration is inhibited by disruption of microtubule function or inhibition of actin polymerization the cell cycle continues resulting in ectopic mitoses. However, if the cell cycle is blocked in the G2-phase by treatment with chemicals the progenitor cells stop their migration and accumulate in the inner area of the ventricular zone (Ueno et al., 2006). This suggests that the accumulation of HCs close to the vitreal side might be caused by inhibited migration due to the G2-phase arrest. Photoreceptors withdraw early from the cell cycle but mature late. Not until E9 do photoreceptors extend an apical process beyond the limiting membrane into the subretinal space. This coincides with the time of HC arrival at the HCL. One might speculate that the HCs need to arrive to a fully organized photoreceptor layer in order to make the correct connections to the PRs. No studies have yet demonstrated ectopic mitoses among the HCs in the mouse retina. However, Lim1 positive cells reside at the vitreal side at E14.5 and do not reach the HCL until E18.5 (Poché et al., 2007). This indicates that even if the mouse HCs do not undergo an extra round of division their migration pattern and timing is similar to that of chicken.

Generation of the horizontal cell subtypes, an unresolved issue

Although one of our objectives was to unravel how the Lim1 and Isl1 populations of HCs are specified in such a controlled manner we are not much closer to an answer. All our attempts at manipulating the genesis of horizontal cells affected both populations equally. Follistatin injections, Ptf1a virus infections and FoxN4 electroporations all generated cells of both subtypes in approximately the 50/50 ratio observed in the wild type retina (Papers I, V and VI). This is in itself an interesting result as it indicates an intrinsic mechanism capable of both keeping track of the cells made and determining subtype fate. Overexpression of Isl1 in HCs residing in the HCL represses endogenous Lim1 expression and causes a subtype fate switch from the H1 morphology to the H2 but not H3 (Suga et al., 2009). Expression of a dominant negative Lim1 variant caused minor morphological changes to the axon and terminal arbors but no subtype fate-switch. Overexpression of Lim1 did not affect the proportion of the HC subtypes. This indicates that a subtype fate-switch can occur in mature HCs caused by Isl1 itself with downregulation of Lim1 as a consequence. Occasionally, cells double labeled for Lim1 and Isl1 were observed during the migration to the vitreal side (Paper II). There may be a onset of Isl1 expression in the Lim1 expressing cells as they are generated causing the subtype fate-switch. However, what may regulate the Isl1 expression is still unknown.
Fgf19 is expressed in chick retina from st16 and the expression closely follows the generation and migration of the Lim1 population (Francisco-Morcillo et al., 2005). Expression of Fgf19 was found in a subpopulation of Prox1 positive HCs but never overlapped with Isl1 labeling (Francisco-Morcillo et al., 2005; Kurose et al., 2004). Further study of factors exhibiting subtype specificity during early development may reveal how HCs regulate their formation of subtypes.

Proliferation of HC-biased progenitors

It is clear that HC-biased progenitors are sensitive to follistatin. The effect of follistatin is likely mediated by its inhibitory effect on activin, which is a signaling molecule that restricts proliferation and induces differentiation. By relieving this restriction to proliferate follistatin allows the HC-biased progenitors to remain in the cell cycle. Initially the treatment with follistatin generated fewer post-mitotic cells but eventually it gave rise to additional HCs (Paper I). Another conclusion was that there has to exist progenitors biased to the HC fate prior to the onset of Lim1. Since we know that the number of Lim1 expressing progenitors was reduced after follistatin injection the progenitor population affected by this must lie further upstream. ACs are also sensitive to activin and follistatin treatment indicating a common progenitor for the HC and AC fate (Moreira and Adler, 2006). One possible explanation why we did not observe any effects on the AC population is the early time of injection and the relatively brief exposure. The quite numerous ACs would require a large effect in order to generate statistically significant quantifiable data. Additionally ACs utilize feedback inhibition and apoptosis to control their final cell number giving room for compensatory events.

Commitment to the horizontal cell fate

FoxN4 and Ptf1a have been shown to be critical for the generation of HCs and ACs (Li et al., 2004; Fujitani et al., 2006). We have shown that their temporal expression coincides with proliferating progenitors that give rise to these populations (Paper IV). We also confirmed the temporal expression pattern that puts FoxN4 upstream of Ptf1a and Prox1. Furthermore, overexpression of both FoxN4 and Ptf1a were sufficient to commit progenitors to HC fate (Paper V and VI). The forced expression of both these genes was also associated with increased apoptosis and disrupted lamination and this may in part explain the migratory defect of the generated HCs.

We and others have shown that Ptf1a is regulated downstream of FoxN4 (Paper V; Li et al., 2004). The increased labeling of FoxN4 observed in progenitors committed to the HC fate likely drives the expression of Ptf1a. The cause of the increase in FoxN4 is still not clear but compiling our results...
with that of other studies suggests that Vsx2, Atoh7 and FoxN4 are key factors in early RPCs. Vsx2 is expressed in the entire early RPC pool. In the zebrafish retina Atoh7 is repressed by Vsx2 and is only expressed in cells that turn off Vsx2 expression (Vitorino et al., 2009). This enables the Atoh7 lineage to give rise to GCs, PRs, HCs, and ACs (Poggi et al., 2005). FoxN4 is also negatively regulated by Vsx2 (Vitorino et al., 2009). Vsx2 may define the early multipotent RPCs which become more restricted as Vsx2 is selectively repressed. When Vsx2 expression was suppressed in the zebrafish retina there was a concomitant upregulation of Atoh7 and FoxN4 whereas the expressions of Crx and Blhl4 were downregulated (Vitorino et al., 2009). This would likely result in an increase of the early generated retinal cells, the GCs and HCs, at the expense of the later generated BPs and rod PRs. These data motivate a model in which a population of cells downregulates Vsx2, resulting in the onset of Atoh7 and FoxN4. In some of these cells the expression of FoxN4 will be maintained or initiated thus driving the expression of Ptf1a. Ptf1a will inhibit transcription of Atoh7 causing these cells to commit to the AC/HC lineage rather than the GC/PR (Fig. 14, Paper V). Further studies are needed to determine how the split within the AC/HC lineage is accomplished.
Figure 14: Commitment to the HC fate. These events should not be considered as cell divisions but rather transition stages with different outcome. Cells that turn off their Vsx2 expression can express Atoh7 and FoxN4. The expression of FoxN4 drives the expression of Ptf1a that inhibits the expression of Atoh7. These events produce progenitors biased to the HC and AC fate.

Future directions
There are several interesting projects currently ongoing in our lab concerning both the effects of FoxN4 overexpression as well as the unusual cell cycle regulation of HCs. Additional electroporations of the FoxN4 vector at various time-points will be needed to give more substance to its involvement in HC and AC fate commitment. The FoxN4 gene might also be cloned into a retrovirus vector (RCAS) that will allow for earlier overexpression. This could determine whether FoxN4 would be able to induce premature neurogenesis in the developing eye. Currently, overexpression of FoxN4 is carried out in retinal neurospheres cultured from the non-pigmented ciliary epithelium and in different retinoblastoma cell lines. We believe that these experiments will clarify the neurogenic ability of FoxN4.
Another priority is the knock-down of FoxN4 and Ptf1a by siRNA. Temporal inhibition of these genes will allow for more precise dissection of their function during fate commitment and subtype formation.

The characterization of the G2-phase arrest in HCs will continue. Alternative pathways that may be responsible for the arrest are currently under investigation. In association with this, there is also interest in the role of HCs in retinoblastoma tumors in light of their ability to re-enter the cell cycle without induction of apoptosis.

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"Remember, licking doorknobs is illegal on other planets!"
Spongebob Squarepants

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References


Li, S., Misra, K., Matise, M.P. and Xiang, M. (2005). FoxN4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord. PNAS 102, 10688-10693


Rompani, S.B. and Cepko, C.L. (2008). Retinal progenitor cells can produce restricted subsets of horizontal cells. PNAS 105, 192-197


Wetts, R. and Fraser, S.E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science, 239, 1142-1145
Zhang, L., Mathers, P H., Jamrich, M. (2000). Function of Rx, but not Pax6, is essential for the formation of retinal progenitor cells in mice. Genesis, 28, 135-142
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