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Xenopus Primary Neurogenesis and Retinogenesis

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ABSTRACT

In the past, significant advances in our understanding of vertebrate neurogenesis have been obtained using the amphibian model system *Xenopus laevis*. Today, *Xenopus* continues to serve as an excellent system to study the molecular mechanisms of neural cell fate determination and differentiation owing to the accessibility of the earliest events of neurogenesis, amenability of the organism to manipulations such as microinjection, electroporation, explant isolation and cultivation. In *Xenopus*, the first neurons are born within the induced neuroectoderm shortly after gastrulation in three longitudinal domains on each side of the midline and are termed primary neurons. In addition to primary neurogenesis, the retina, in which six classes of neuronal cells and the Müller glial cell sequentially differentiate, also serves as an outstanding system to study the molecular events of neurogenesis. In this review we will detail the current knowledge of events that control neuronal and glial cell fates in *Xenopus*, with an emphasis on intrinsic factors, cell cycle regulators and the Notch pathway in the context of primary neurogenesis and retinogenesis.

Keywords: cell fate determination, Notch-Delta, primary neurogenesis, proneural genes, retina, *Xenopus*

CONTENTS

PRIMARY NEUROGENESIS IN *XENOPUS* 26
Neural induction 26
Early neural genes 27
Primary neurogenesis 27
Proneural genes 28
Bridging the early neural and proneural genes 29
Lateral inhibition 29
Primary neurogenesis and the cell cycle 30
Specificity of the proneural genes 30
Outlook 30
NEURAL AND GLIAL CELL FATE DETERMINATION IN THE RETINA: CONTRIBUTION OF THE FROG 30
Histogenesis in embryonic and adult *Xenopus* retina 30
Birthdate of retinal cell types during *Xenopus* embryogenesis 30
Post-embryonic histogenesis in amphibians 30
Retinal histogenesis: mechanistic role of environmental cues and cellular competence 31
Intrinsic factors governing neural cell fate decisions 33
bHLH and homeodomain families of transcription factors 33
Post-transcriptional regulation 33
Gliogenesis in *Xenopus* retina 33
Outlook: *Xenopus* as a model to investigate retinal subtype specification 34
Diversity among neuronal cell types 34
Early biases in amacrine subtypes revealed in *Xenopus* 34
ACKNOWLEDGEMENTS 34
REFERENCES 34

PRIMARY NEUROGENESIS IN *XENOPUS*

In *Xenopus*, similar to other vertebrate anamniotes, neurogenesis occurs in two phases. The first neurons, termed primary neurons, are born shortly after gastrulation and are responsible for the early movements and responses of the larvae (Roberts 2000). Due to the simplicity and accessibility of primary neurogenesis, *Xenopus* is a valuable model system to elucidate the cascade of events that controls early vertebrate neurogenesis. Secondary neurogenesis occurs later at the tadpole stage and more closely correlates with neurogenesis in amniotes. As the majority of the genes implicated in primary neurogenesis are also expressed during secondary neurogenesis, it is anticipated that most, if not all, of the molecular events are conserved (Wullimann *et al.* 2005).

Neural induction

The initial step in the establishment of the vertebrate nervous system is the decision of the ectodermal cells to adopt a neural at the expense of an epidermal fate (reviewed in De

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Invited Review

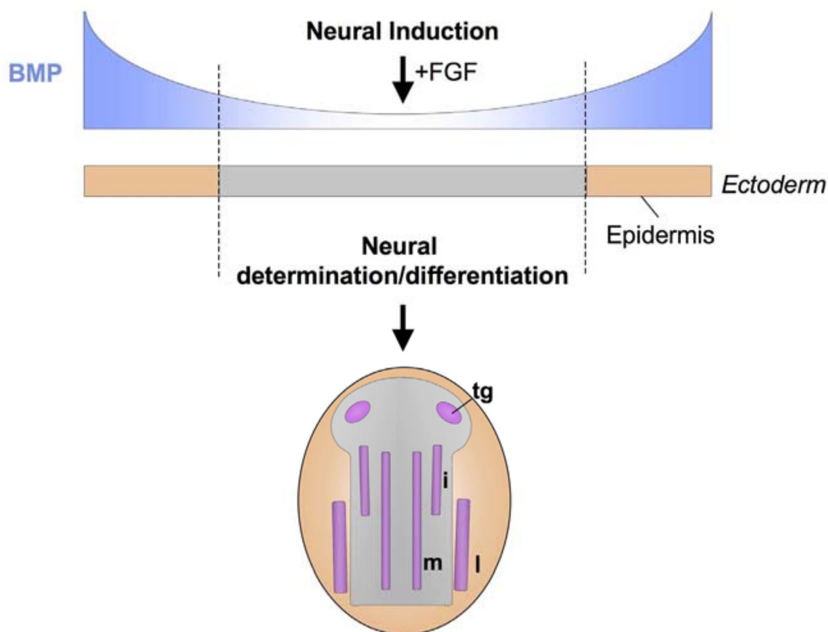

Fig. 1 Neural induction and the domains of primary neurogenesis. A variety of mechanisms are present in the gastrula embryo, which inhibits BMP signaling in the presumptive neural ectoderm, thereby contributing to the establishment of the neural plate. In addition to BMP inhibition, low levels of FGF signaling are also required for neural induction. Within the large domain of the induced neural plate, only a subset of these cells will undergo neuronal differentiation at this stage. These primary neurons are established in a lateral (l), medial (m) and intermediate (i) longitudinal domain on each side of the midline in the posterior region of the embryo. An additional site of neuronal differentiation at this time is the trigeminal ganglion (tg).

Robertis and Kuroda 2004). During neural induction and demarcation of the neural plate, ectodermal cells are endowed with the capacity to become neural precursors. One of the main driving forces of a neural fate is the inhibition of Bone Morphogenetic Protein (BMP) signaling, which is achieved through multiple mechanisms (**Fig. 1**). BMP activity is inhibited extracellularly in pregastrula and gastrula stage embryos by factors secreted from the blastula Chordin- and Noggin-expressing center (BCNE) and the Spemann Organizer, respectively (Kuroda *et al*. 2004). BMP signaling is also attenuated by the phosphatase Dullard, which promotes the dephosphorylation and degradation of BMP receptors (Satow *et al*. 2006). Fibroblast growth factor (FGF) and insulin growth factor (IGF) signaling further inhibit the BMP pathway by downregulating Smad1 activity, an intracellular transducer of BMP signaling (Richard-Parpaillon *et al*. 2002; Pera *et al*. 2003). In addition, canonical Wnt signaling inhibits BMP at the transcriptional level (Baker *et al*. 1999). However, BMP inhibition is not sufficient for neural induction in vertebrates (Launay *et al*. 1996; Sasai *et al*. 1996). In chick (Wilson *et al*. 2000), zebrafish (Kudoh *et al*. 2004) and more recently in *Xenopus*, it has been demonstrated that low levels of FGF signaling are required for the development of neural fates, independent of the role of FGF as an inhibitor of BMP signaling (Delaune *et al*. 2005). This initial neural induction phase gives rise to tissue of anterior character, which is subsequently transformed by posteriorizing signals such as retinoic acid and members of the FGF and Wnt families (reviewed in Gould and Grainger 1997).

Early neural genes

Several prepattern genes have been identified that are positively regulated by the events of neural induction giving rise to their broad expression throughout the presumptive neural ectoderm of the early gastrula embryo (reviewed in Moody and Je 2002). Many of these genes contribute to the establishment and stabilization of a neural fate. Members of the *SoxB1* family of HMG box transcription factors (*Sox2* and *Sox3*) have a role in maintaining the early neural state and serve as markers of proliferating neural precursor cells (Mizuseki *et al*. 1998a; Kishi *et al*. 2000). In general, cells that express *Sox3* do not express markers of neuronal differentiation indicating that Sox3 may promote proliferation at the expense of differentiation (Bourguignon *et al*. 1998; Bellefroid *et al*. 1998). However, a direct interaction of Sox3 with the cell cycle machinery has not been established. The Zic family of zinc finger proteins also plays a critical role in the initial phase of neural development in *Xenopus*. While *Zic1-3* are all expressed broadly throughout the prospective neural ectoderm of early gastrula stage embryos, they differ functionally. Overexpression of *Zic1*, *Zic2* or *Zic3* expands the neural ectoderm, while *Zic1* and *Zic3* induce neuronal differentiation, and *Zic2* inhibits this process (Nakata *et al*. 1997; Mizuseki *et al*. 1998a; Brewster *et al*. 1998). *Zic2* becomes localized in the posterior region of the neural plate in longitudinal domains that alternate with stripes of proneural gene expression (Brewster *et al*. 1998). This expression pattern has implicated Zic2 in restricting the territories of primary neurogenesis (discussed below).

SoxD and members of the Iroquois homeobox family (*Xiro*1-3) are late stabilizing genes that maintain the undifferentiated state. Their overexpression positively regulates the proneural genes at the open neural plate stage, although this may be indirect, at least for Xiro1, through *BMP4* inhibition (Gomez-Skarmeta *et al*. 2001). In addition, despite their ability to promote a neural fate, they prevent differentiation of primary neurons until tailbud stages (Bellefroid *et al*. 1998; Gomez-Skarmeta *et al*. 1998; Mizuseki *et al*. 1998b; Yeo and Gautier 2005). The inhibition of neuronal differentiation by Xiro1 may be the result of *Zic2* and *Hairy2A* induction, concomitant with inhibition of the *growth-arrest-and-DNA-damage-induced gamma* gene (*XGadd45-γ*) (de la Calle-Mustienes *et al*. 2002). Taken together, these data suggest that *SoxD* and *Xiro1-3* contribute to the expansion of the neurogenic domain while delaying neuronal differentiation.

Primary neurogenesis

The induced neural ectoderm is comprised of undifferentiated mitotically active cells, from which only a restricted number of cells will exit the cell cycle and commence differentiation (Hartenstein 1989). Primary neurogenesis first occurs posteriorly in three longitudinal domains on each side of the dorsal midline and can be visualized by the general neuronal differentiation marker, *neuron-specific class II β-tubulin* (*N-tubulin*) (Oschwald *et al*. 1991; Moody *et al*. 1996) (**Figs. 1, 3**). An additional site of neurogenesis at the open neural plate stage is the trigeminal ganglion neurons. In the region of the embryo anterior to the midbrain-hindbrain boundary, neuronal differentiation is delayed until after closure of the neural tube (Papalopulu and Kintner 1996).

The neural primordium at the open neural plate stages consists of a bilayered epithelium, which is generated by orientated cell divisions during blastula stages (Hartenstein 1989; Chalmers *et al*. 2003). Primary neurons arise from

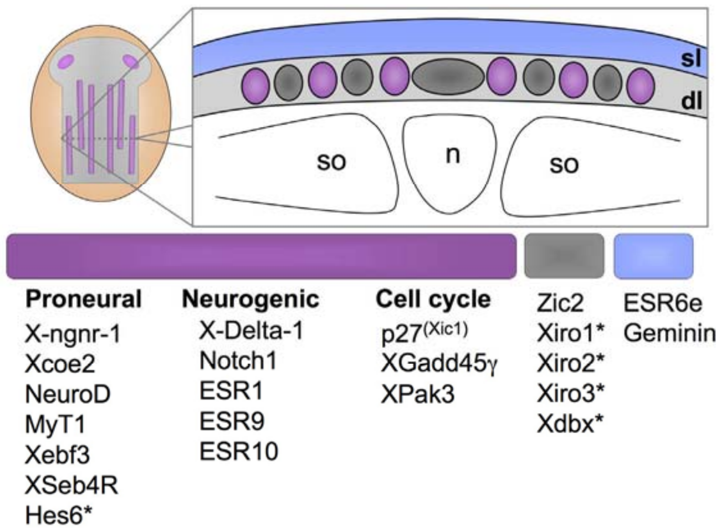

Fig. 2 Expression of positive and negative regulators of primary neurogenesis. The primary neurons arise from the deep layer (dl) of the neuroectoderm, in which several proneural genes are expressed, as well as cell cycle inhibitors (shown in purple). Early acting genes are expressed earlier and in broader domain than later acting genes (Bellefroid *et al*. 1996). Within the territories of primary neurogenesis, the neurogenic genes limit the number of cells that undergo neuronal differentiation. Neuronal differentiation is restricted to discrete domains, in part due to the activity of inhibitors present in the inter-neuronal stripes (dark grey). Other neuronal inhibitors are expressed in the superficial layer (sl; shown in blue) that presumably render it refractory to neuronal differentiation at the open neural plate stage. Asterisks indicate proteins that are only partially expressed within the domains. so: somites; n: notochord.

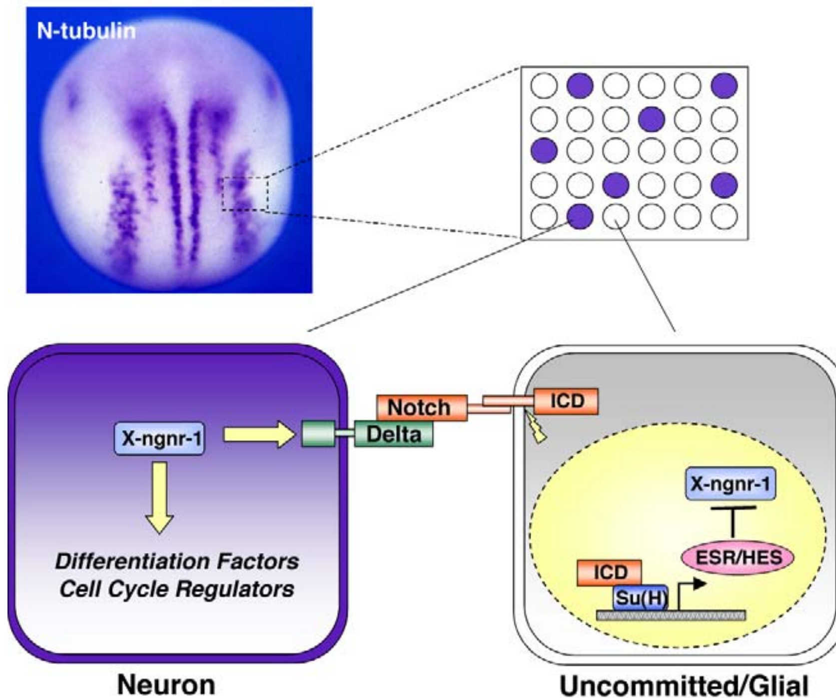

Fig. 3 Lateral inhibition mediated by the Delta-Notch Pathway. As shown in the whole mount *is situ* hybridization of the general neuronal marker *N-tubulin*, neurons arise in the longitudinal domains in a speckled pattern. This pattern is the result of lateral inhibition mediated by the cell to cell signaling Delta-Notch pathway. In the cell fated to become a neuron, the neuronal determination transcription factor X-ngnr-1 not only activates those genes required for differenttiation, but also induces the ligand *X-Delta-1*. X-Delta-1 binds to the Notch receptor on the neighboring cells, inducing a series of proteolytic cleavages that releases the intracellular domain of Notch (NICD). NICD then participates in the activation of repressor bHLH proteins that inhibit both the transcription and function of the proneural genes, thereby maintaining the undifferentiated state of the cell.

precursors within the deep layer (sensorial), while secondary neurons arise equally from those in both the superficial layer (epithelial) and deep layer (Hartenstein 1989). Accordingly, general neural markers such as *Sox3* and *SoxD* are expressed in both layers, and in contrast, the proneural transcription genes *neurogenin related-1* (*X-ngnr-1*) and *NeuroD* are present only in the deep layer (Chalmers *et al*. 2002). The superficial layer is refractory to neuronal differentiation induced by a variety of regulators including *X-ngnr-1*, *NeuroD* and *FGF-8*. The precise molecular mechanism that confers resistance to the superficial cells remains unknown, but may in part be due to the presence of inhibitory factors. The enhancer-of-split related bHLH genes *ESR6e* and *XHes2* are expressed exclusively in the superficial layer, and their misexpression in the deep layer inhibits neuronal differentiation (Chalmers *et al*. 2002; Sölter *et al*. 2006). Throughout late gastrula stages, the coiled-coil gene *Geminin* is present in both layers of the neuroectoderm, but expression is eliminated from the deep layer prior to terminal differentiation of the primary neurons (Seo *et al*. 2005a). As the presence of Geminin blocks neuronal differentiation, it has been proposed that Geminin may act as a timer to control the transition from proliferation to differentiation (Seo *et al*. 2005a). Functionally, this may occur through modulation of chromatin structure *via* interaction with the SWI/SNF chromatin-remodeling complex, which is required for transcriptional activity of the bHLH proneural genes (Seo *et al*. 2005a, 2005b).

As the neural folds rise and fuse to form the closed neural tube, the two layers interdigitate into a monolayer of cells that give rise to different classes of neurons dependent on their position in the neural tube (Hartenstein 1989). The medial, intermediate and lateral longitudinal domains of primary neurons will occupy a ventral to dorsal sequence and give rise to motor, inter-, and Rohon-Beard sensory neurons, respectively (Chitnis *et al*. 1995; Roberts 2000).

Proneural genes

The differentiation of the primary neurons is driven by proneural transcription factors, which promote a cascade of cell-specific transcription factors and genes, required for the cell fate determination and withdrawal of the progenitor cells from the cell cycle. Genetic and molecular studies have identified several genes that function as key regulators in converting the induced neuroectoderm into fully differentiated neurons. In *Xenopus*, many of these proneural genes are expressed in a pattern that closely resembles *N-tubulin*, defining a synexpression group (**Fig. 2**). One class of proneural genes is homologous to genes of the *Drosophila atonal* and *achaete-scute* complex. Members of this basic helix-loop-helix (bHLH) family in *Xenopus* include

X-ngnr-1 (Ma *et al*. 1996), *NeuroD* (Lee *et al*. 1995) and *Xath3* (Takebayashi *et al*. 1997). In addition, members of the *Ebf/Olf-1* family, *Xcoe2* (Dubois *et al*. 1998) and *Xebf3* (Pozzoli *et al*. 2001), the zinc finger protein *MyT1*, as well as the RNA binding protein *XSeb4R* (Boy *et al*. 2004), function as positive regulators of neurogenesis. The sequential activation of the proneural genes in the domains of primary neurogenesis represents the successive stages of determination and differentiation. A variety of studies demonstrate that *X-ngnr-1* is at the top of this cascade, operating as a determination factor with other components acting downstream or in parallel (Ma *et al*. 1996). Already at gastrula stages, *X-ngnr-1* expression prefigures the territories of primary neurogenesis, earlier than other known proneural genes (Perron *et al*. 1999). Moreover, X-ngnr-1 is sufficient to instruct the non-neural ectoderm to undergo neuronal differentiation (Ma *et al*. 1996).

The proneural bHLH proteins heterodimerize with ubiquitously expressed bHLH proteins (E-proteins) *via* the HLH domain (reviewed in Quan and Hassan 2005). Through interactions mediated by the basic domain, the heteromeric complex then binds to E-box sequences (CANNTG) in regulatory regions of target genes and activates their transcription. The ability of bHLH proteins to readily heterodimerize also permits inhibition of their activity through the interaction with other proteins, including bHLH repressors of the E(spl)/Hairy/Her family (discussed below). Additional negative regulators that function at the protein level are members of the Inhibitor of Differentiation (Id) family. These proteins harbor a HLH domain allowing protein dimerization, but lack the basic domain necessary for DNA-binding (Yokota 2001). Members of this family act as effective inhibitors of *X-ngnr-1* and *NeuroD* proneural activities in explant assays (Liu and Harland 2003). However, an endogenous role in the context of *Xenopus* primary neurogenesis has yet to be defined.

Bridging the early neural and proneural genes

One intriguing question is how the discrete territories of primary neurogenesis domains are selected within those of the broadly expressed prepattern genes. This may in part be attributed to the presence of negative regulators (**Fig. 2**). The homeodomain-encoding gene *Xdbx* is expressed in the neural plate between the medial and intermediate domains of primary neurogenesis (Gershon *et al*. 2000). A role for *Xdbx* in restricting neuronal differentiation is underscored by its ability to inhibit *X-ngnr-1* and *N-tubulin* following its overexpression in *Xenopus* embryos. *Zic2* may also contribute to this process, particularly at late neurula stages where it is expressed in the posterior region of the neural plate in domains that alternate with the longitudinal domains of the proneural genes (Brewster *et al*. 1998). Finally, *XETOR*, a member of the *MTG/ETO* family expressed in the discrete domains of primary neurogenesis, has been proposed to participate in the refinement of the proneural domains size (Cao *et al*. 2002).

To fully understand how the stereotypical pattern of primary neurogenesis is specified, it will also be necessary to elucidate those factors and pathways that are required downstream of the immediate targets of neural induction and prior to the action of the proneural genes. The MEK5-ERK5 pathway was demonstrated to be required during neurogenesis downstream of *SoxD* and upstream of *X-ngnr-1* (Nishimoto *et al*. 2005). Furthermore, *FGF-9* and *FGF-13* were identified as growth factors whose expression was induced by SoxD (Nishimoto *et al*. 2005). A role for FGF signaling in promoting neurogenesis is further illustrated by the abundance of ectopic neurons obtained upon *FGF-8b* overexpression in *Xenopus* embryos (Hardcastle *et al*. 2000). The bHLH transcriptional repressor *Xmxi1*, which is expressed earlier and in broader expression domains as compared to *X-ngnr-1*, was also found to be an essential component of the neurogenesis cascade functioning prior to *X-ngnr-1*, but downstream of *SoxD* (Klisch *et al*. 2006).

Lateral inhibition

Due to the activation of lateral inhibition, only a subset of cells in the domains of primary neurogenesis undergo neuronal differentiation giving rise to the scattered pattern of *N-tubulin* (Chitnis *et al*. 1995; Ma *et al*. 1996). This cell to cell signaling cascade mediated by the Delta-Notch pathway (**Fig. 3**) enables cells that initially express higher levels of proneural genes to escape from lateral inhibition and differentiate as neurons, while maintaining the adjacent cells in an undifferentiated neural precursor state (reviewed in Wang and Barres 2000). Indeed, in the cell fated to become a neuron, X-ngnr-1 induces the transcription of downstream genes involved in the differentiation network, but also activates *X-Delta-1* and *X-Serrate-1* expression (Chitnis *et al*. 1995; Kiyota *et al*. 2001). These single-pass membrane ligands interact with the extracellular domain of the Notch receptor located on the neighboring cell. This binding triggers a series of proteolytic cleavages of the Notch receptor that releases the Notch intracellular domain (NICD) (Kadesch 2004). The *cis*-endocytosis of X-Delta-1 may also play an important role in the processing of Notch (reviewed in Kiyota and Kinoshita 2004). Released NICD enters the nucleus and associates with the DNA binding protein suppressor of hairless (Su(H)), converting it from a transcriptional repressor to an activator. This ultimately leads to inhibition of neurogenesis (**Fig. 3**). Accordingly, overexpression of constitutively activated Notch (NICD) or X-Delta-1 inhibits the production of primary neurons (Coffman *et al*. 1993; Chitnis *et al*. 1995). Conversely, dominant-negative versions of the *X-Delta-1*, *Notch*, or *Su*(*H*) increase the density of neurons within the discrete territories of primary neurogenesis when overexpressed (Chitnis *et al*. 1995; Wettstein *et al*. 1997; Kiyota *et al*. 2001).

The best characterized direct targets of Notch signaling are related to the *Drosophila* bHLH transcriptional repressor proteins encoded by the *Enhancer of split E*(*spl*) *complex* and *Hairy* genes. The E(spl)/Hairy/Her proteins can directly inhibit both the transcription of proneural genes and their protein activity, thereby antagonizing differentiation (Dawson *et al*. 1995). Several genes of this class, including *ESR1*, *ESR9*, and *ESR10*, are indeed expressed in the territories of primary neurogenesis and are activated by Su(H)-dependent Notch signaling (Wettstein *et al*. 1997; Koyano-Nakagawa *et al*. 1999; Li *et al*. 2003). In line with this, their overexpression inhibits neuronal differentiation. However, not all members of the E(spl)/Hairy/Her family antagonize neurogenesis. *Hes6* is inhibited by Notch signaling and activated by X-ngnr-1 in *Xenopus* embryos (Koyano-Nakagawa *et al*. 2000). Consistent with its regulation, *Hes6* overexpression promotes neuronal differentiation in the territories of primary neurogenesis, providing a positive feedback loop. As the function of Hes6 is independent of DNA binding, its proneural activity may be related to its ability to antagonize hairy-like repressors through heterodimerization (Koyano-Nakagawa *et al*. 2000). The *Notch regulated ankyrin repeat* (*Nrarp*) (also known as *XNAP*) gene may be an additional direct target of Notch signaling, but acts to provide a negative feedback control (Lahaye *et al*. 2002). *Nrarp* is expressed in similar territories to *X-Delta-1* and *Notch* and forms a ternary complex with NICD and XSu(H), thereby negatively modulating their activities (Lamar *et al*. 2001).

The proneural zinc finger transcription factor *MyT1*, which is positively regulated by *X-ngnr-1* and negatively by Notch, plays an essential role in allowing neuronally fated cells to escape lateral inhibition and enter terminal differentiation (Bellefroid *et al*. 1996). As *MyT1* overexpression does not affect *X-Delta-1* nor *X-ngnr-1* expression, its ability to overcome lateral inhibition is not likely to directly interfer with the lateral inhibition machinery. Rather, cells would escape lateral inhibition even when the lateral inhibitory machinery is active, from the moment when a

certain threshold of MyT1 level is reached (Bellefroid *et al*. 1996).

Altogether these data highlight the complex interplay between the neural bHLH proteins and the lateral inhibitory machinery. How a balance is struck to ensure that the proper number of neurons is produced is however still unclear. One mechanism that may be important to establish how cells are initially selected from an initial background of proneural activities is the different requirements of histone acetyltransferase (HAT) activity of X-ngnr-1 target genes. X-ngnr-1-mediated transcriptional activation of *X-Delta-1* has a lower dependence on HAT activity than that of the proneural genes *MyT1* and *NeuroD*, and may therefore have a lower threshold of activation. This may enable the genes in the lateral inhibition pathway to be induced prior to the genes that promote differentiation (Koyano-Nakagawa *et al*. 1999).

Primary neurogenesis and the cell cycle

Neuronal differentiation requires the interplay between cell cycle regulation and cell fate determination. It is well established during vertebrate neurogenesis that neural cell fate is regulated by cell cycle progression and *vice-versa* that determination/differentiation factors participate in the regulation of the cell cycle (reviewed in Ohnuma and Harris 2003).

In the anterior region of the early neurula *Xenopus* embryo, neuronal differentiation is in part suppressed by XBF-1, a Fox transcription factor that maintains active cell division *via* the inhibition of the Cip/Kip cyclin-dependant kinase inhibitor *p27Xic1* (Hardcastle and Papalopulu 2000). The homeobox transcription factor Xrx1 also represses *p27Xic1* anteriorly, and in addition, induces the antineurogenic transcription factors *Xhairy2* and *Zic2* (Andreazzoli *et al*. 2003). Conversely, several genes have been identified which are expressed in the territories of primary neurogenesis and support neuronal differentiation through the induction of cell cycle arrest, including *p27Xic1*, the *p21 activated kinase 3* (*XPak3*), and *XGadd45-γ* (de la Calle-Mustienes *et al*. 2002; Souopgui *et al*. 2002; Vernon *et al*. 2003). *p27Xic1* is not induced by X-ngnr-1, but is required in the cascade of neuronal differentiation between *X-ngnr-1* and *NeuroD* (Vernon *et al*. 2003). *XPak3* and *XGadd45-γ* may act later than p27Xic1 as both are activated by X-ngnr-1, as well as the later acting differentiation factor NeuroD (de la Calle-Mustienes *et al*. 2002; Souopgui *et al*. 2002). While knock-down of either p27Xic1, XPak3 or XGadd45-γ with antisense morpholino oligonucleotides inhibits primary neurogenesis, only loss of XPak3 and p27Xic1 results in an increased proliferation of the neural plate, suggesting partial redundancy (de la Calle-Mustienes *et al*. 2002; Souopgui *et al*. 2002; Carruthers *et al*. 2003). Cell cycle arrest is a prerequisite for cells to undergo neuronal differentiation, but it is not sufficient. Through the use of *XPak3* and *p27Xic1* deletion mutants, the ability of these factors to induce cyclin-dependent kinase inhibition could be uncoupled from their activity on neuronal differentiation, suggesting additional activities for these regulators during neurogenesis (Souopgui *et al*. 2002; Vernon *et al*. 2003). Accordingly, p27Xic1 has been proposed to have a role in stabilizing X-ngnr-1 protein levels (Vernon *et al*. 2003).

Specificity of the proneural genes

While many genes have been identified that promote neurogenesis, few studies have been performed that compare the selectivity of downstream genes induced by the proneural factors or their ability to specify neuronal subtypes. *X-ngnr-1*, *Xath3* and *Xebf2* all induce ectopic sensory neurons in *Xenopus* embryos (Perron *et al*. 1999; Pozzoli *et al*. 2001), suggesting their ability to promote specific neuronal programs. In line with this idea, Tallika and collaborators compared in ectodermal explants, the temporal pattern of a limited set of downstream target genes induced by *X-ngnr-1* and *Xash1,* respectively (Talikka *et al*. 2002). While both genes activated the expression of the late neuronal marker *N-tubulin* with a similar time course, each induced distinct patterns of early downstream target genes, reinforcing the view that the different proneural genes may act to drive the formation of specific neuronal cell populations.

Outlook

As many of the regulators of primary neurogenesis that have been identified thus far are transcription factors, one can expect that they directly participate in the transcriptional regulation of other proneural genes resulting in a temporal cascade of gene activation. In fact some degree of linearity exist in the hierarchy of the proneural genes, for example X-ngnr-1 activates *NeuroD* directly and not *vice-versa* (Ma *et al*. 1996; Perron *et al*. 1999). However, several genes that are expressed later can activate early expressed genes. These positive feedback mechanisms, coupled with the existence of shared functional domains, such as the bHLH motif, have hindered the elucidation of the epistatic relationship between many of the proneural genes. The lack of our understanding in the control of neuronal differentiation is further underscored by the fact that direct activators of neuronal differentiation markers have not yet been identified. There is also only limited knowledge on the role of post-transcriptional and post-translational regulatory mechanisms in the process of primary neurogenesis. Taken together, despite the identification of multiple genes participating in vertebrate neurogenesis, the molecular interactions and signaling networks linking proliferation, specification, determination and mitotic withdrawal of the neural progenitors into discrete neuronal subtypes remains to be fully elucidated.

NEURAL AND GLIAL CELL FATE DETERMINATION IN THE RETINA: CONTRIBUTION OF THE FROG

Histogenesis in embryonic and adult *Xenopus* retina

Birthdate of retinal cell types during Xenopus *embryogenesis*

Retinal histogenesis in mammalian species lasts for weeks to months and follows an invariant temporal scheme. The ganglion cells are the first to be born, followed by amacrine cells, cones, horizontal cells, rods, bipolar cells and ultimately Müller glia. This histogenic process is largely compressed in *Xenopus* embryos. Tritiated thymidine injections throughout the stages of retinogenesis reveals that more than 95% of the embryonic retinal cells are born within a 25 hour period (from stage 24 to stage 41), resulting in a large overlap of neurogenesis in the different layers (Holt *et al*. 1988). Nevertheless, experiments precisely examining the retinal birth sequence in the frog have demonstrated the same order of genesis as compared with mammalians (Rapaport *et al*. 1996). Irrelevant of the species, these *in vivo* birthdates are conserved in explants or dissociated retinal cell cultures, strongly suggesting the existence of a temporal control of retinoblast determination.

Post-embryonic histogenesis in amphibians

The retina of amphibians, like that of fish, continues to grow postembryonically and throughout life, due to the addition of new cells originating from the ciliary marginal zone (CMZ). This region, located at the peripheral edge of the retina, is a continuously proliferative neuroepithelium (**Fig. 4**). The CMZ has the outstanding feature of displaying a spatial organization that mirrors all developmental stages of retinogenesis. Self-renewing stem cells are confined to the most peripheral region while differentiating retinal

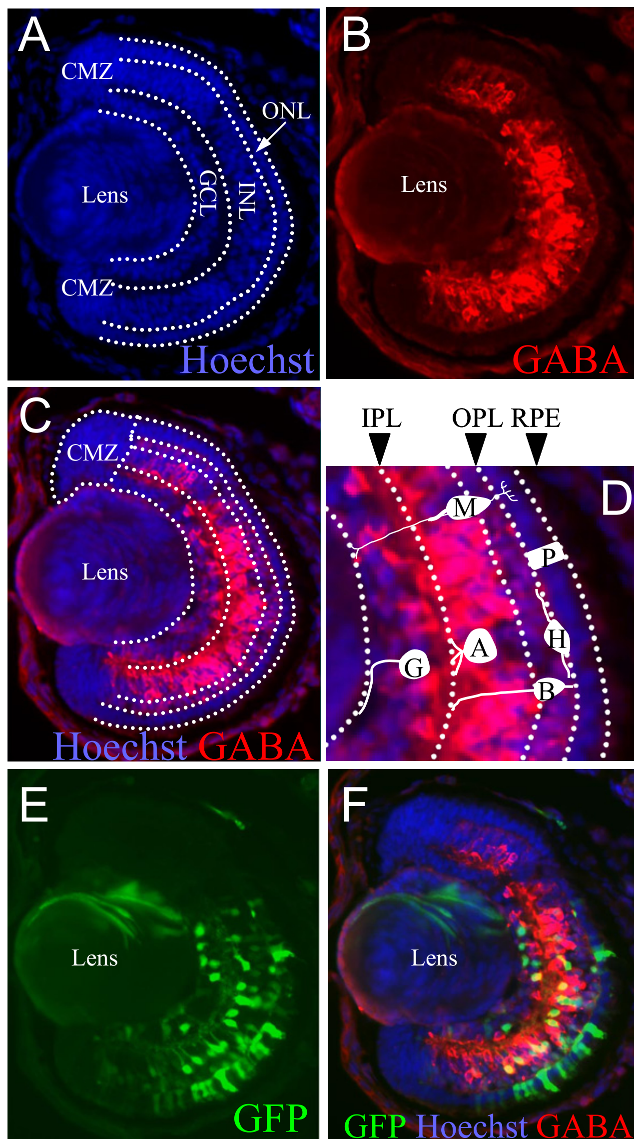

Fig. 4 Organization of the *Xenopus* retina. (A) Example of Hoechst nuclear labeling highlighting the three nuclear layers and the CMZ of a stage 41 *Xenopus* tadpole retina. The ganglion cell layer (GCL) is composed of ganglion (G) and displaced amacrine (A) cells; the inner nuclear layer (INL) comprises amacrine (A), bipolar (B), horizontal (H) and Müller glial (M) cells and the outer nuclear layer (ONL) is the photoreceptor (P) layer. (B-D) Immunostaining with a GABA antibody (B) that stains amacrine and horizontal cells, counterstained with Hoechst (C, D). GABA staining allows to subdivide the INL into an inner and an outer part. (D) Higher magnification of (C) with schematic positioning of the different cell types across the retina. CMZ: ciliary marginal zone; IPL: inner plexiform layer; OPL: outer plexiform layer; RPE: retinal pigment epithelium. (E) Typical cross section of a stage 41 retina showing cells expressing GFP, following lipofection at the neurula stage of a GFP expression plasmid in the presumptive retina. (F) The morphology of labeled cells as well as their position in the different nuclear layers (whose visualization is here aided by Hoechst and GABA staining) allows their identification.

progenitors are located more centrally (Wetts *et al.* 1989; Dorsky *et al.* 1995; Perron *et al.* 1998). Such an organization that recapitulates the temporal order of retinal embryonic histogenesis has proved to be a powerful system to gain insight into the genetic cascade controlling retinogenesis. The tadpole CMZ (Dorsky *et al.* 1995, 1997; Harris and Perron 1998; Perron *et al.* 1998; Ohnuma *et al.* 1999), as well as the metamorphic CMZ (Casarosa *et al.* 2005), can be subdivided in four distinct zones according to the relative expression patterns of several genes, including components of the Notch pathway, bHLH and homeodomain transcription factors, and cell cycle regulatory components (**Fig. 5**). Thus, the zone where a gene is expressed provides a first clue to position it in the genetic hierarchy controlling retinogenesis. Moreover, coexpression of distinct genes inside of a particular domain allows the prediction for functional relationships, which can be subsequently tested and that in some cases have now been demonstrated (see below).

Another advantage of amphibian retina that may help uncover the mechanisms sustaining neurogenesis is their regeneration capacity. In urodele amphibians like the newt, complete retina and lens regeneration occurs throughout their lives (reviewed in Okada 1980). In contrast, anuran amphibians have been thought to maintain this capacity only in the larval stage, while losing it following metamorphosis. However, Yoshii et al. recently reported that the neural retina regenerates following its surgical removal in the mature anuran amphibian, *Xenopus laevis*. The neural retina regenerates both from transdifferentiation of the retinal pigment epithelial cells and from differentiation of stem cells in the CMZ (Yoshii *et al.* 2007). These findings offer a novel experimental model to investigate the molecular regulatory network involved in retinogenesis in physiological and pathological conditions.

Retinal histogenesis: mechanistic role of environmental cues and cellular competence

Following specification of the eye field, retinal precursor descendants progressively generate the different retinal cell types. Lineage experiments using retroviral infection in rodents (Turner and Cepko 1987; Turner *et al.* 1990) or tracer injection of single cells in the *Xenopus* optic cup (Holt *et al.* 1988; Wetts and Fraser 1988) demonstrated the multipotency of retinal progenitors towards any type of retinal cell. In addition, the apparent randomness in the clones cellular composition suggested that non-lineage-dependent mechanisms are involved in specifying cell identity (Holt *et al.* 1988). This led to the hypothesis that the local environment has an instructive role in cell fate decisions. Indeed, studies in *Xenopus* and other systems have shed light on the importance of extrinsic factors in the regulation of retinal cell determination (reviewed in Cepko 1999). Signaling from postmitotic neurons has been shown to modulate cell fate decisions of retinoblasts. For example, selective ablation of monoaminergic amacrine cells in the frog retina results in overproduction of these cells by retinal progenitors (Reh and Tully 1986). These data suggest that such lineages are sensitive to feedback by inhibitory environmental signals. Many diffusible signals have been found to influence histogenesis in the retina (reviewed in Agathocleous and Harris 2006). Misexpression of the growth factor FGF-2 affects the proper ratio of the various retinal cell types, particularly photoreceptors (McFarlane *et al.* 1998; Patel and McFarlane 2000). Accordingly, lack of photoreceptors is observed in transgenic tadpoles expressing a dominant negative FGFR-4a under the control of a promoter that is active in retinal progenitors (Zhang *et al.* 2003). This is consistent with the idea of an endogenous FGF signal influencing cell fate decisions in the developing vertebrate retina. Another example of extrinsic signaling affecting retinoblast decisions is the cell to cell signaling mediated by the Delta-Notch pathway. Experiments in the frog retina demonstrated a role for Delta-Notch signaling in regulating cell competence through inhibition of progenitor differentiation (Dorsky *et al.* 1995; Dorsky *et al.* 1997; reviewed in Rapaport and Dorsky 1998; Perron and Harris 2000). Misexpression of an activeted form of Notch "freezes" progenitors in an undifferentiated, neuroepithelial state (Dorsky *et al.* 1995). Conversely, progenitors where Notch signaling is blocked from early stages through overexpression of a dominant negative form of Delta, escape from lateral inhibition and adopt early retinal fates, *i.e.* cones and ganglion cells (Dorsky *et al.* 1997). In contrast, inhibition of Notch signaling at later stages biases cells towards later cell types i.e. rod photoreceptors, demonstrating the importance of timing in the function of Delta. This led to a model where retinal progenitors, which

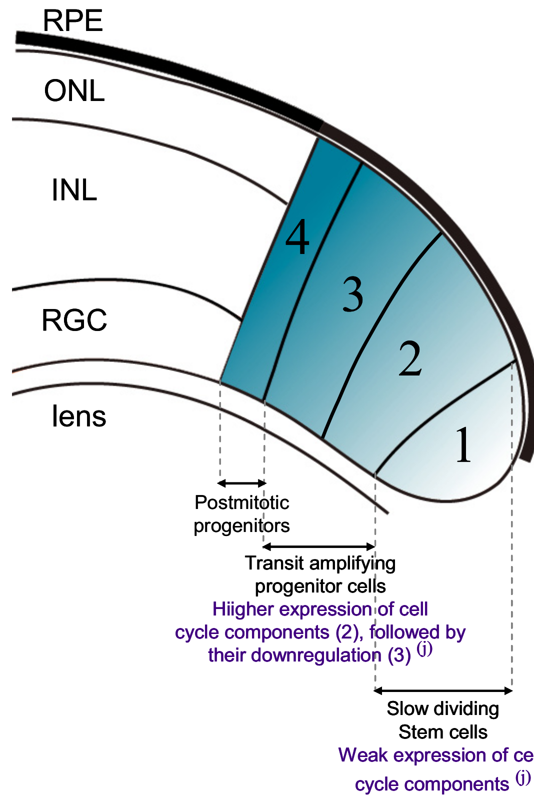

Fig. 5 Gene expression patterns in the *Xenopus* CMZ. Schematic representation of the different zones in the CMZ, based on the expression of various genes as indicated in the table. mRNAs of these genes were detected either only in zone 1, from zone 2 to 4, in zones 3 and 4, in all the CMZ or only in zone 4. Double *in situ* hybridizations for genes expressed in zone 1 have not been performed yet, preventing us to know whether they overlap in zone 2 or not. Note that Vax2 is only expressed in the ventral CMZ (Casarosa *et al*. 2005). References are under parentheses: (a) Perron *et al*. 2003; (b) our unpublished data; (c) Perron *et al*. 1999; (d) Perron *et al*. 1998; (e) Sölter *et al*. 2006; (f) Casarosa *et al*. 2005; (g) van Raay *et al*. 2005; (h) Boy *et al*. 2004; (i) Amato *et al*. 2005; (j) Ohnuma *et al*. 2002a.

	1	2/3/4	3/4	1/2/3/4	4
Transcription factors	Gli3 (a) Gli2 (a) Hairy1 (b) Hairy2b (b)	X-Ngnr-1 (c) Xash1 (d) Xash3 (d) HRT1 (b) ESR1 (d) ESR3 (d) ESR9 (b) XHes2 (e) Vax2 (f) Xchx10 (f)	Xath3 (d) Xath5 (d) NeuroD (d) XMyT1 (d) Xotx2 (d)	Pax6 (d) XRx1 (d) XOptx2 (d)	
Ligands Receptors	Smo (a)	Notch (d) Delta (d) Frizzled5 (g)			
RNA binding proteins	Xrp1 (b)	XSeb4R (h)		Nrp-1 (i)	ElrC (i) BruL-1 (i)

change their competence over time, use lateral inhibition mediated by Delta-Notch to generate the different retinal cell types in appropriate numbers at the right stage of development.

Despite the growing wealth of data on the nature of extrinsic factors and feedback mechanisms involved in retinal determination, no instructive environmental cues affecting particular fates have been identified so far. Rather, it is now established that environmental signals can alter the relative proportions of each cell type generated at a given time, but cannot influence progenitors to make temporally inappropriate cell types. Evidence comes from heterochronic transplant experiments, some of which have been performed in amphibians. Cells from young *Xenopus* embryonic retinas were dissociated and grown together with those from older embryos, and the timing of photoreceptor birth assayed. Young cells appeared uninfluenced by older cells, expressing photoreceptor markers on the same time schedule as when cultured alone (Rapaport *et al*. 2001). A similar result was obtained *in vivo* by heterochronic grafts of optic vesicle plugs from young embryos into older hosts (Rapaport and Dorsky 1998; Rapaport *et al*. 2001). Thus, retinal progenitors intrinsically acquire the ability to respond to photoreceptor-inducing cues by a mechanism that runs on a cell autonomous schedule. Such findings in various species led to the development of the "competence model" of retinal development, which proposes that progenitors pass through intrinsically determined competence states, during which they are capable of giving rise to a limited subset of cell types under the influence of extrinsic signals (reviewed in Livesey and Cepko 2001). The model predicts that the conserved order of histogenesis is based in part on an intracellular timer that controls the appropriate expression of intrinsic cues responsible for successive changes in competence (discussed

below).

Intrinsic factors governing neural cell fate decisions

bHLH and homeodomain families of transcription factors

Classical assays of putative determination factors in *Xenopus* widely used blastomere mRNA injection for overexpression studies or misexpression of dominant-negative/ positive form of candidate genes. However, this technique is not always suitable for investigating gene function in the context of retinogenesis as early effects often preclude correct interpretation of later phenotypes observed in the retina. To bypass this limitation, Holt *et al*. developed the lipofection technique (**Fig. 4**) whereby targeted transfection of a limited number of precursors in the presumptive retina allows for clonal analysis of gene perturbation effects during retinal development, without affecting early eye morphogenesis (Holt *et al*. 1990). Lipofection also offers the possibility of loss-of-function experiments through the transfection of morpholino oligonucleotides into retinoblasts (Ohnuma *et al*. 2002b). This technique has undoubtedly contributed to build the widely accepted model stipulating that basic helix-loop-helix (bHLH) activators, in combination with homeodomain factors, are responsible for neuronal cell type specification (reviewed in Hatakeyama and Kageyama 2004).

The first bHLH misexpression in the *Xenopus* retina was *Xath5* (Kanekar *et al*. 1997). This *Drosophila atonal*-related gene promotes ganglion genesis when overexpressed *in vivo*. Consistent with this data, is the almost complete depletion of retinal ganglion cells (RGCs) in zebrafish and mouse *Xath5* mutants (Brown *et al*. 2001; Kay *et al*. 2001; Wang *et al*. 2001). Xath5 may push retinal precursors towards a ganglion cell fate by directly regulating the expression of RGC differentiation factors. Several direct targets of Xath5, which may account for its effects, have been identified, including transcriptional regulators (*XBrn3d*, *Xebf2*, *Xebf3*, *XETOR* and *NKL*), an RNA binding protein (*elrC*) and a cell cycle component (*XGadd45-γ*) (Hutcheson and Vetter 2001; Vetter and Brown 2001; Logan *et al*. 2005). Similar to *Xath5*, misexpression of bHLH genes *Xath3* and *X-ngnr-1* biases progenitors towards early cell types at the expense of late born cells (Perron *et al*. 1999). However, these various bHLH factors display some differences in their respective effects on each cell type, suggesting that in addition to their general proneural activity they may contribute to some aspect of neural cell type specification in the retina (Perron *et al*. 1999).

Another set of transcription factors affecting retinal cell fate belongs to the superfamily of homeodomain proteins (HD). In *Xenopus*, *XOtx5b* is expressed in both photoreceptors and bipolar cells, while a closely related member of the same family of transcription factors, *XOtx2*, is expressed in bipolar cells only. Lipofection of retinal precursors with *XOtx5b* biases them toward photoreceptor fates, whereas a similar experiment with *XOtx2* promotes bipolar cell genesis (Viczian *et al*. 2003). *Xvsx1*, which is expressed in retinal progenitors and bipolar cells (D'Autilia *et al*. 2006), has also recently been shown to support bipolar cell determination (Decembrini *et al*. 2006). Another example is *Xbh1*, which has proved to play a crucial role in retinal cell determination, acting as a switch towards ganglion cell fate (Poggi *et al*. 2004).

The observation that several transcription factors may be expressed in the same progenitors has raised the possibility of a combinatorial mode of action. This question has been extensively addressed in *Xenopus* through experiments where each of six retinally expressed bHLH transcription factors (NeuroD; X-ngnr-1; Xath3; Xath5; Xash1; Xash3) were coexpressed in retinal progenitors with each of eight retinally expressed HD transcription factors (XRx1; XOptx2; XSix3; XPax6; XOtx2; XOtx5b; XBH; XChx10) using lipofection (Wang and Harris 2005). The effects of each combinations were assayed on the six major cell types in the retina and demonstrated possible intrinsic combinatorial coding.

Post-transcriptional regulation

While these data demonstrate the crucial role of homeobox and bHLH genes in retinal cell identity, they do not address the question of how the neurogenetic timing is controlled. Moore et al. found that the timing of bHLH function relies on posttranslational regulation, allowing bHLH factors with overlapping expression to function independently. Specifically, NeuroD function in the retina can be inhibited by glycogen synthase kinase 3β (GSK3β), while Xath5 function can be inhibited by Notch (Moore *et al*. 2002). Thus, the varying context in which bHLH proteins act may be crucial to drive their effect on specific retinal cell types. Recently, Decembrini and collaborators demonstrated that the three *Xenopus* HD transcription factors *Xotx5b*, *Xvsx1* and *Xotx2* are initially transcribed, but not translated in early retinal progenitors (Decembrini *et al*. 2006). Interestingly, their translational onset coincides with photoreceptor (*Xotx5b*) and bipolar cell (*Xvsx1* and *Xotx2*) birth. Perturbation of cell cycle progression experiments suggest that decreased cell cycle duration that normally occurs in late progenitors, is necessary to remove translational inhibition of these genes (Decembrini *et al*. 2006). These data strongly support the idea that a retinal cell clock measures cell cycle length, rather than the absolute time spent in division, to provide regulatory cues authorizing genesis of a particular cell type at the appropriate moment. Translational inhibitors are presumably part of such a clock machinery, but their molecular nature is at present unknown.

RNA-binding proteins and micro-RNAs may be good candidates to play such key roles in the post-transcriptional regulation of gene expression during retinogenesis. We recently described the expression patterns of different neural RNA binding proteins (RBPs) in the developing and post-embryonic *Xenopus* retina (**Fig. 5**). Distinct spatio-temporal expression was found for RBPs belonging to different families (Amato *et al*. 2005). This is reminiscent of previously described cell-type-specific expression profiles of transcription factors and suggests that these post-translational regulators may be crucially involved at multiple steps of retinogenesis. Accordingly, we demonstrated that the RBP XSeb4R exerts a proneural role in the developing retina downstream of the bHLH factor NeuroD (Boy *et al*. 2004). A great challenge is now to assess other RBPs functions and identify their targets to gain new insights into the genetic network governing retinal cell determination.

Gliogenesis in *Xenopus* retina

In contrast to the large amount of data concerning neuronal cell type specification, mechanisms sustaining the generation of glial *versus* neuronal cells remain more ambiguous. Müller cells, the principal glial population in the retina, are generated from the same pool of multipotent stem cells that give rise to retinal neurons. Recent studies have shed light upon the extracellular and intracellular signaling pathways that regulate Müller glial cell genesis.

Accumulating evidence supports the involvement of cell cycle components in the cell fate determination process and conversely, molecules originally thought to have a primary role in cell determination, have been shown to influence cell proliferation (reviewed in Ohnuma *et al*. 2001; Ohnuma and Harris 2003; Cremisi *et al*. 2003). A surprising inducer of glial fate in the *Xenopus* retina was found to be p27Xic1, a cyclin-dependent kinase inhibitor (CDKI) of the Cip/Kip subfamily. Expression of p27Xic1 progressively increases as retinal histogenesis proceeds. Its overexpression enhances the generation of Müller cells, while lipofection with an antisense construct reduces by half their percentage (Ohnuma *et al*. 1999). Thus, it is likely that the gradual increase

of p27Xic1 in the developing retina not only limits the number of retinal cells but also increasingly favors the fate of the last cell type to be born in the retina, the Müller glia. The CDKIs p16Xic2 and p17Xic3 share similar properties in Müller cell fate specification (Daniels *et al*. 2004). Finally, Ohnuma and collaborators demonstrated that p27Xic1 gliogenic activity necessitates an active Delta-Notch pathway, in accordance with the observation that Müller cells are the last cells in the *Xenopus* retina to express Notch1 (Ohnuma *et al*. 1999).

Consistent with the above results, is the finding that components of the Notch pathway are gliogenic. In the *Xenopus* retina, lipofection of retinal progenitors with XSu(H)Ank, a constitutively active form of the Notch mediator Suppressor of Hairless, leads to accelerated cell cycle exit and concomitant increase of Müller cells (Ohnuma *et al*. 2002a). Although it remains to be determined whether Notch has an instructive role in *Xenopus* gliogenesis as it has been recently proposed in rodents (Tomita *et al*. 1996; Bao and Cepko 1997; Furukawa *et al*. 2000), its ability to inhibit expression and activity of proneural bHLH genes (reviewed by Artavanis-Tsakonas *et al*. 1999) surely contributes to favor glial *versus* neuronal cell fate. This is achieved through activation of bHLH transcriptional repressors of the E(spl)/Hairy/Her family. We recently identified a novel member of this family in *Xenopus*, *XHes2*, which inhibits glial cell genesis when knocked down, while dramatically biases retinal precursors towards a glial fate when overexpressed (Sölter *et al*. 2006). We showed that the gliogenic activity of XHes2 relies on its ability to inhibit neuronal differentiation by at least two distinct mechanisms: it not only negatively regulates *X-ngnr-1* and *NeuroD* transcription, but it also physically interacts with a subset of proneural bHLH proteins. The next step will be to identify other transcription factors that act downstream or in collaboration with XHes2, as well as target genes that function to repress neurogenesis or to promote gliogenesis in the retina.

Outlook: *Xenopus* as a model to investigate retinal subtype specification

Diversity among neuronal cell types

The different neuronal cell types in the retina are still divided in cellular subtypes, based on morphological and biochemical criteria, and according to established connections. More than twenty types of amacrine cells and many classes of ganglion cells have been identified, although the functional relevance of such diversity is far from being explained (MacNeil and Masland 1998). Mechanisms sustaining the specification of the different neuronal subclasses are largely unknown. Harris and Messersmith showed that two cellular inductions are involved in photoreceptor determination during development in the retina (Harris and Messersmith 1992). The first is responsible for biasing cells toward either a generic photoreceptor or a cone fate, while the second directs cells toward a rod cell fate. The prevalent model, based on this data plus others in rodents (Bramblett *et al*. 2004; Chow *et al*. 2004) thus proposes that the competence of a precursor is restricted in a step-wise manner so that the determination process initially leads to a generic cell type and subsequently specifies the subtype.

Early biases in amacrine subtypes revealed in Xenopus

The above model can be questioned due to the extensive lineage experiments performed in the laboratory of Moody and collaborators (Huang and Moody 1995, 1997; reviewed in Zaghloul *et al*. 2005). Indeed, some retinal subtypes seem to be specified as soon as the early cleavage stage. Transplantations experiments of individual blastomeres demonstrate that some of them are intrinsically biased to produce specific neurotransmitter subtypes of amacrine cells (Moody *et al*. 2000). These data have contributed to refine the previous vision of retinal cell fate being independent of lineage mechanisms. It now seems that early lineage biases account for the determination of at least certain cell subtypes and that progenitors of the eye field are presumably mosaic, consisting of both multipotent stem cells and fate-restricted progenitors (reviewed in Zaghloul *et al*. 2005; see Cayouette *et al*. 2006 for more general discussion of retinal lineage biases). In the course of the discovery of the underlying molecular cues involved in this process, Zaghloul and Moody (Zaghloul and Moody 2007) recently found that two transcription factors expressed during the formation of the eye field, Rx1 and Pax6, may be involved in early amacrine neurotransmitter subtypes specification in *Xenopus*. It is therefore tempting to re-evaluate the implication of other factors, known to be involved in retinal cell fate decision, focusing more closely on their effects on retinal subtypes specification.

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