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Developmental and Genetic Control of Cell Number in Retina

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Now, nearly a century after Weismann, it is self-evident that the missing chapters of the Modern Synthesis—the merging of genetics with development and the merging of development with evolution—remain the major tasks before us.

Leo W. Buss (1987)

Introduction

The retina is one of the most highly conserved parts of the central nervous system, with unambiguous homology of major cell types among most chordates. These cells and their progenitors therefore share common patterns of gene activity. This widespread commonality is illustrated most dramatically by the *Pax6* genes—a small family of transcription factors that trigger the formation of retinas, ommatidia, and photoreceptors in animals as diverse as fruit flies, squid, ascidians, fish, frogs, mice, and humans (Gehring and Ikeo 1999; Onuma et al. 2002).

Retinas differ strikingly, however, in terms of numbers and distribution of cells. Structural diversity of the vertebrate retina is generated using a relatively constant palette of cell types (Walls 1942). Downstream effects mediated by transcription factors such as *Pax6*, and a rapidly expanding list of trophins, hormones, cytokines, and other signaling molecules can produce significant changes in numbers and ratios of cells and their interconnections. Evolution of the retina has therefore involved the episodic modulation of developmental processes that often adjust quantities, not quality.

The contrast between universal mechanisms and structural diversity highlights the need to study development using complementary approaches. The first approach focuses on fundamental qualitative features that are common denominators; the second approach focuses on quantitative adaptations of genetic networks that underlie functional and evolutionary transformations, as well as individual differences. This review is divided into sections that consider developmental, genetic, and functional factors that modulate types and numbers of cells in the vertebrate retina from these two complementary viewpoints.

There are approximately 65 to 70 distinct cell types in retina (Marc and Jones 2002; Marc, 2003 this volume), each with its own idiosyncratic developmental history, and we only consider key themes and a few exemplar cell types. In Part 1, we cover the initial stages of visual system development in which cell proliferation and differentiation produce the cellular substrates common to all vertebrate retinas. Most of our examples are drawn from studies of the South African clawed frog, *Xenopus laevis*, a long-favored experimental subject for developmental analyses. In Part 2, we consider the relation between cell number and visual system performance, addressing the question of how much variation is tolerated within species and why. Finally, we describe a novel approach called complex trait analysis that is being used to uncover the genetic basis of individual differences in retinal development. Combined developmental and genetic approaches should eventually provide a much better understanding of how evolutionary

modifications have produced such amazing arrays of almost perfectly adapted eyes and retinas, a subject that gave Darwin serious pause (and a cold chill) a century and a half ago (Darwin 1899; Dawkins 1996).

Part 1: The generation of a menagerie of retinal cells

A wide variety of retinal cell types are generated in appropriate numbers by sequential and cooperative changes in patterns of gene expression that are coupled with cell division, cell differentiation, and cell death. These changes start at very early stages, even before gastrulation and the establishment of the neural plate, and changes continue through to late stages of fetal development. Differentiation and cell division are directed by factors that act at different spatial scales and stages, but both ultimately affect the activity of enhancer and promoter response elements in single cells. Broadly speaking, these factors include 1) inherited maternal determinants, 2) regional molecular gradients, 3) cell autonomous lineage factors, and 4) local intercellular signals. In this section we consider some of the key mechanisms involved in selecting the embryonic progenitors of the retina, partitioning part of the forebrain neuroectoderm into a field of specified retinal stem cells, and the progressive differentiation of the many cell types common to all vertebrate retinas that are eventually derived from these stem cells.

Molecular complexity of retina. The adult retina expresses at least 25,000 transcripts from over 20,000 genes (Sharon et al. 2002). Even more genes are likely to be expressed throughout development. The majority of these genes undoubtedly have common roles in cellular differentiation and metabolism, but a significant fraction—including many so-called housekeeping genes—are likely to contribute to unique features of retinal development and function. This is demonstrated clearly by the fact that over 10% of 12,422 transcripts quantified in triplicate using microarrays (Affymetrics U74Av2) differ more than 2-fold in abundance between retina and other brain regions such as cerebellum, brainstem, and forebrain (see databases at www.webqtl.org/search.html). Even with modest numbers of samples, the expression levels of well over 20% of retinal transcripts differ significantly from forebrain transcripts using standard statistical criteria at the $p < 0.05$ level.

Given the imposing molecular complexity of development of the eye and retina, the reliability of the process is astonishing. The robust molecular systems that ensure the production of a functional retina clearly are built on a principle of genetic redundancy and feedback interactions. While a few genes are essential for eye development (e.g., *Pax6*), most genetic processes are sensitive to context and can adapt to significant perturbations. The inactivation of important genes is often associated with only mild ocular and retinal effects. For example, the loss of the retinoic acid alpha receptor early in retinal development has essentially no effect on the inner retina despite early and intense expression among the pool of progenitors (Mori et al. 2001; Zhou et al. 2001). A process that is controlled by a complex developmental network involving genetic and epigenetic interactions is likely to produce greater variance and flexibility, and is also likely to be more resistant to environmental perturbations (Waddington and Robertson 1966). As presented below, flexibility is built into the retinal developmental program from beginning (progenitor competence) to end (cell type specification).

The first step: selecting competent retinal progenitors

Retinal development can be divided into several steps, some of which occur even before overt morphogenesis of the eye cup (Figure 1). First, a subset of the pluripotent embryonic cells acquires the competence to contribute to the retina. From this competent pool, a smaller subset is biased to become the retina-forming cells. Later, a specified retinal stem cell population emerges from the descendants of the biased embryonic cells to form the eye field in the anterior neural plate. Further interactions during neural tube morphogenesis segregate this population into three major compartments: neural retina, pigmented retina and optic stalk. Each of these compartments goes on to produce different subsets of specialized cells with distinct functions in the mature eye.

An initial question is, how and when during do development pluripotent embryonic cells acquire the differential competence to contribute to the retina? Is this a partly stochastic process, or do early factors bias or restrict the selection of retinal-forming embryonic progenitors? *Xenopus* embryos have been crucial for studying the roles of maternal determinants, lineage effects, and cell-cell inductions at this early stage (even before gastrulation) because eggs and blastomeres are large and easily manipulated (Moody, 1999). Each retina in *Xenopus* descends from a stereotypic subset of nine animal blastomeres at the 32-cell stage (Figure 2), and each of these blastomeres produces characteristic proportions of the cells that make up the mature retina (Huang and Moody 1993). Are these nine cells the only cells that are competent to form the retina, and just how fixed is their commitment to a retinal fate?

These questions have been largely answered by transplanting single cells to novel positions at a very early stage when only maternally inherited transcripts are expressed. The first key finding is that even at this stage, not all blastomeres are equally competent to contribute to the retina. For example, vegetal blastomeres transplanted to the most retinogenic coordinates never contribute progeny to retina (Huang and Moody, 1993). This developmental restriction could not be overcome by providing components of the known maternal signaling pathways involved in neural and dorsal fate specification (see below), even under situations in which ectopic heads and eyes were induced successfully (Moore and Moody 1999). These results indicate that vegetal blastomeres contain one or more maternal molecules that repress the transcription of genes that are critical in the initial steps of retinal differentiation.

Other blastomeres are not so refractive. Both ventral animal blastomeres and equatorial blastomeres that normally do not contribute progeny to the retina can reprogram when transplanted to the center of the retinogenic zone (Huang and Moody 1993). Furthermore, if the most retinogenic blastomere is deleted, a ventral implant will be respecified in response to interactions with neighboring cells to help produce a normal-sized retina (Huang and Moody 1993). Complementing these findings, the retina-forming blastomeres are biased but not completely committed. When transplanted to a ventral vegetal site that normally produces gut and tail, they retain their neural fate but fail to make retina (Gallagher et al. 1991). Collectively, these embryonic manipulations demonstrate that the correct cellular coordinates within the animal hemisphere are necessary from the earliest stage for a blastomere to produce its normal cohort of mature retinal cells.

The position-specific selection of retina-forming blastomeres from the competent pool appears to be mediated by the local signaling environment within the blastula. The ectopic expression of components of several growth factor pathways involved in embryo patterning, such as activin, fibroblast growth factor (FGF), and bone morphogenetic protein (BMP), demonstrate that competent blastomeres acquire the ability to express a retinal fate by being located in an environment in which BMP signaling is repressed (Moore and Moody 1999). Expression of BMP4 in a blastomere that normally is a major contributor to the retina inhibits its retinal fate, whereas repressing BMP signaling in a blastomere that normally gives rise to epidermis induces retinal cells within that lineage. Consistent with this model in frog, *Bmp7* null mice are anophthalmic (Dudley et al. 1995) and rat embryos cultured in anti-BMP7 antibodies have reduced or absent eyes (Solursh et al. 1996). The inhibition of BMP signaling in the dorsal part of the embryo defines the domain of presumptive retina, just as it defines the domain of presumptive neural plate (Harland 2000).

Another important question is whether the competent pool of retina-forming embryonic cells is invariant in size, or responsive to extrinsic pressures. Although fate maps show that there are nine retina-forming blastomeres in *Xenopus* (Fig. 2), in fact not every one of these cells contributes to retina in every embryo. In the initial map (Moody 1987), the major progenitors contributed to retina 85–100% of the time, whereas the minor progenitors contributed only 20–50% of the time. Thus, there is surprising individual variation in the number of embryonic cells that produce the retinal lineage. There also is individual variation in the number of retinal cells descended from each retina-forming blastomere (Huang and Moody 1993). For the major progenitor, there is a 2-fold range in descendants between embryos, but for the others there is as much as a 10-fold range. These data have been verified in many subsequent studies using similar techniques and demonstrate that the ultimate numbers of progeny produced by each lineage are not predetermined, and individual variation is well tolerated within consistent limits. This is apparently just as true in mammals as in *Xenopus* (Williams and Goldowitz 1992a; Goldowitz et al. 1996).

The second step: specifying stem cells

The next step in retinogenesis is to select from the competent pool those cells that will become specified as retinal stem cells (Figure 1). After the neural plate is established, it is subdivided into fields that give rise to different CNS domains. Both retinas arise from a common anterior midline group of cells called the eye field, which is specified as the sole source of retinal cells when the neural plate is established (Saha and Grainger 1992; Perron and Harris 1999).

A number of transcription factors regulate pattern formation in the anterior neural plate and eye field, and they are crucial in determining whether neuroepithelial cells express a retinal fate as opposed to another anterior neural fate. *Otx2*, a vertebrate homologue of the *Drosophila orthodenticle* gene, has at least two potential functions in the formation of the eye field. Early *Otx2* expression in the anterior endoderm appears necessary for the induction of the anterior neural plate (Acampora et al. 1995; Ang et al. 1996; Rhinn et al. 1998) and its continued expression is required to initiate or maintain the expression of several other key neurogenic factors (Rhinn et al. 1998), leading to the regionalization of

the neural plate that allows the eye field to form. *Bf1,* a fork head/winged helix transcription factor, is expressed in the forebrain and in the future nasal retina (Tao and Lai 1992; Hatini et al. 1994). The *Xenopus* homologue *Fkh4,* is expressed in the anterior margin of the neural plate and then in the eye field (Dirksen and Jamrich 1995). *Pax6,* the potent paired box and homeodomain transcription factor mentioned in the Introduction, is highly expressed in the eye field, and its ectopic expression causes the formation of ectopic eye structures in fly and frog (Halder et al. 1995; Altmann et al. 1997; Chow et al. 1999; Kenyon et al. 2001). *Rx1*, a novel homeobox gene, is expressed exclusively in the eye field, retina, and pineal gland (Casarosa et al. 1997; Mathers et al. 1997; Furukawa et al. 1997). Its overexpression in *Xenopus* causes enlargement of native eye tissue and ectopic patches of pigmented retina (Mathers et al. 1997; Kenyon et al. 2001). *Six3*, related to the *Drosophila sine oculis* gene, is expressed in the eye field and optic vesicle (Oliver et al. 1995). Overexpression of mouse *Six3* in fish or mouse causes ectopic retinal tissues to form (Loosli et al. 1999; Lagutin et al. 2001). Another *six* family gene, *Optx2,* is also expressed in the eye field and optic vesicle (Toy et al. 1998), and when *Optx2* is overexpressed it transforms more caudal neural plate into eye field (Bernier et al., 2000). Loss-of-function mutations of *Bf1*, *Otx2*, *Pax6,* and *Rx1* each lead to loss of retinal tissue, indicating that all are likely to have important roles during establishment of the eye field.

The segregation of the anterior neural plate into eye field versus non-eye field domains is likely to result from the localized expression of several transcription factors, including those described above, which then initiate eye-specific transcriptional programs. In addition, some of these genes are involved in even earlier retinal fate decisions. *Otx2, Pax6,* and *Rx1* are expressed more widely and diffusely at stages just before the formation of the eye field, and they can cause ventral epidermal-forming blastomeres to contribute to retina by altering gastrulation movements that allow their descendants access to the eye field (Kenyon et al. 2001). Thus, these genes appear to regulate the selection of retinal stem cells at multiple levels: transcriptional modulation, signaling, and cell movements.

This complex program of selecting retina-specific stem cells provides several opportunities for adjustments in cell number. From the pregastrula competent pool of cells, only a subset forms the eye field. Expressing the correct genetic program increases the probability of competent cells being selected to become retinal stem cells. For example, in the experiments of Kenyon et al. (2001) not every cell that ectopically expressed the exogenously supplied retinal gene expressed a retinal fate. Some cells migrated into the eye field and others did not. Some of the ventrally derived retinal clones were composed of hundreds of cells and some were composed of fewer than ten cells. These data indicate that there are local cues that also influence these fate decisions. Subtle epigenetic variations in these cues would have significant effects on final retinal cell numbers by altering the size of the initial stem cell pool.

The third step: specification of different cell types

After the compartmental domains of the future eye are defined during neural plate and neural tube stages, cells within the optic cup begin to differentiate into the many different cell types that characterize the functional vertebrate retina. They exit the cell cycle in a defined spatio-temporal pattern, migrate to appropriate layers, turn on the correct

neurotransmitters or visual pigments, and develop the correct pre- and post-synaptic connections. The ganglion cells project long axons to very distinct targets, whereas intrinsic cells make highly specialized connections locally. Intricate choreography! A large number of factors and interactions affect the diversity and numbers of distinct retinal cell types that are generated during this period of retinogenesis (reviewed in Harris 1997; Cepko 1999; Livesey and Cepko 2001; Vetter and Moore 2001; Vetter and Brown 2001).

Roles of environmental and lineage in cell fate. The optic cup was one of the first vertebrate tissues in which the issue of lineage regulation of cell phenotype specification was addressed. Vertebrate embryos, with their enormous numbers of cells, are difficult subjects for complete lineage studies because it is not technically feasible to map every mitotic division. Nonetheless, parts of retinal lineages have been defined by intracellular injection of tracers into proliferating cells of the optic vesicle/cup (Holt et al. 1988; Wetts and Fraser 1988) or by injecting a recombinant retrovirus that infects proliferating cells and marks their clones (Turner and Cepko 1987; Turner et al. 1990). Both techniques showed that dividing cells of the optic vesicle/cup are multipotent; labeled clones spanned the entire thickness of the retina and were composed of various combinations of cells. They also retain striking pluripotency right up to their last division, often producing daughters of two very different cell types, for example, Muller glial cells and bipolar cells (Turner and Cepko 1987). The great majority of retinal stem cells are not committed to produce only a single phenotype, nor do they appear to be restricted to an identifiable invariant combination of cell types.

With the exception of retinal astrocytes, that originate from separate progenitors in the optic stalk (Chang-Ling and Stone 1991), it was initially thought that lineage restriction plays little if any role in determining types or numbers of retinal cells. However, the amazing diversity in the cellular composition and size of clones labeled at very early stages (Williams and Goldowitz 1992a) suggested the presence of matched heterogeneity among retinal progenitors which implies some form of differential commitment of the progenitors (Williams and Goldowitz 1992b, Goldowitz et al. 1996). The original lineage tracing techniques could not label single identified progenitors across different animals— a criterion by which lineage mechanisms are usually identified, and this left the original data open to alternate interpretations.

Multiple retinal stem cell types. In fact, we now know that the eye field stem cells are a heterogeneous population (Moody et al. 2000, Dyer and Cepko 2001). There are at least two kinds of cells, those that produce radial clones and those that produce layer-restricted clones. In *Xenopus* these two progenitors differ in the layer distribution of their constituents, their cellular complexity, and the number of cell divisions remaining in their respective lineages. These two different eye field stem cell types may represent either two different determinative states or separate lineages for the early-formed primary (layered) versus later-formed secondary (radial) retina, similar to what has been described for neural plate progenitors of primary and secondary spinal neurons (Hartenstein 1989). Two kinds of clones (single cells and radial columns) also have been described in the early chick and mouse retina (Fekete et al. 1994; Reese et al. 1999). Cellular diversity in type and number could easily be generated by changes in the processes that separate the original stem cell pool into different subsets of progenitors that have different ultimate

fates. This could result in correlated shifts in populations that participate in common visual function—a primary example being scotopic and photopic subsystems.

Although lineage studies show that progenitor cells in the optic vesicle and cup are multipotent, recent *in vitro* studies also suggest that this pool contains differentially biased precursors (Jensen and Raff 1997; Alexiades and Cepko 1997; Marrow et al. 1998; Belleveau and Cepko 1999). In fact, the concept that retinal lineages are not homogeneously multipotent but are differentially biased was elegantly demonstrated by a statistical analysis (Williams and Goldowitz 1992b) of published data (Turner et al. 1990); the frequency of clones containing only two cell types was much higher than expected and that the frequency of clones extending across all layers was much lower than expected. Furthermore, *in vitro* studies showed that some rat optic cup progenitors are biased to produce amacrine and horizontal cells (Alexiades and Cepko 1997). Other progenitors appear biased to produce either rod or bipolar cells (Marrow et al. 1999).

One model put forward to reconcile strong evidence of extrinsic control of retinal cell differentiation and strong evidence of fate-biased determination acting via committed progenitors, is simply that retinal progenitors pass through a series of determinative states, each of which is intrinsically specified to respond to particular environmental cues that influence the cell types produced (Cepko 1999). For example, embryonic retinal progenitor cells differ in many characteristics from neonatal ones (Watanabe and Raff 1990; Marrow et al. 1998; Lillien and Cepko 1992; Waid and McLoon 1995; Alexiades and Cepko 1997), and these states can be influenced by the presence of other cell populations (Reh and Tully 1986; Reh 1992; Austin et al. 1995; Waid and McLoon 1998; Belleveau and Cepko, 1999) and cytokines (Harris, 1997). An important aspect of this process is a cell autonomous "clock" that regulates the competence of progenitors (Rapaport et al. 2001). Subtle changes in timing of the determinatives states and/or progenitor competence could be significant contributors to individual variation in cell numbers and types.

Lineage bias of some amacrine subtypes. One approach to obviate the problem of labeling progenitors at random was to map the origins of specific cell types from the "identified" retina-forming blastomeres of the 32-cell frog embryo (Figure 2). When specific progeny within retinal clones were identified by neurotransmitter expression, certain subtypes of amacrine cells dopamine (DA), neuropeptide Y (NPY), serotonin (5-HT) were observed to arise from different subsets of blastomeres, whereas others (GABA, glycine) did not (Huang and Moody 1995, 1997). This novel information likely was revealed because blastomeres can be consistently identified, unlike single progenitors in the optic cup, and each subtype of cell may follow its own subprogram of developmental instructions that would not be obvious from studying the broad population. This proposed lineage bias of DA, NPY and 5-HT amacrine cell specification could result from the asymmetric distribution of intrinsic (i.e., maternal) factors that autonomously influence the different amacrine fates, or from inductive signaling related to the position in which the blastomere descendants differentiate. To test whether amacrine fate is intrinsically biased, blastomeres with distinct amacrine fates were transplanted to a position that normally expresses a different amacrine fate (Moody et al. 2000). A lateral animal blastomere expressed its normal, large number of DA amacrine cells after it was transplanted into a position that normally produces few of these cells,

suggesting an intrinsic bias in amacrine fate within this lineage. However, another blastomere changed amacrine fate in accord with its new position. These experiments illustrate that the retina-producing blastomeres are already a mosaic of intrinsically biased and positionally specified progenitors.

At later stages of development, there also is evidence for lineage bias in the specification of amacrine cell subtypes. When DA, NPY, and 5-HT amacrine cell subtypes first differentiate, they are arranged as single cells scattered across the inner nuclear layer (Huang and Moody 1995, 1997). As more cells differentiate, the first cells are joined by like-expressing cells to form small clusters (Huang and Moody, 1995, 1997). If amacrine cell clusters were induced to express the same neurotransmitter by local interactions with their peers, then clusters should form even in the absence of cell division and after the emergence of the first amacrine cell. This is true of 5-HT clusters (Moody et al., 2000). In sharp contrast, DA and NPY clusters are virtually eliminated by blocking mitosis, indicating that cells in these clusters are generated by continued asymmetrical divisions of a strongly biased lineage. An important point is that even within a single cell class, specification of subtypes can be modulated by surprisingly different processes and at different stages of development.

Local intercellular signals. Local signaling in the developing retina is critical in the progressive changes in retinal progenitors and in the subsequent differentiation of many cell types (Adler and Hatlee 1989; Repka and Adler 1992; Reh 1992; Gan et al 1996, 1999, Harris 1997). A growing number of secreted factors have effects on cellular differentiation, usually as assayed in tissue culture and explants. CNTF (Fuhrmann et al. 1995; Kirsch et al. 1996; Ezzeddine et al. 1997), BDNF (Rickman and Rickman 1996), GDNF (Politi et al. 2001), TGFá (Lillien 1995), retinoic acid and thyroid hormone (Kelley et al., 1994, 1995), SHH (Levine et al. 1997), FGFs (Pittack et al. 1991, 1997; Opas and Dziak 1994; Hyer et al. 1998; McFarlane et al. 1998), and BMPs (Belecky-Adams and Adler 2001): all appear to have important roles in discriminating among different retinal class fates, in some cases also shifting relative numbers of early and late generated cell types. Activin signaling, for example, decreases the number of photoreceptors and increases non-photoreceptor cells present in low-density retinal cultures in a dose-dependent manner (Davis et al. 2000). Retinoic acid can do the opposite (Kelley et al. 1994). Chick ganglion cells express BMP receptors and retinal explants exposed to BMPs contain enhanced numbers of ganglion cells (Carri et al. 1998). FGF2 has been reported to either stimulate (Hicks and Courtois, 1992) or suppress (Perron and Harris 1999) photoreceptor numbers. There is convincing evidence that in the intact embryo FGF2 may not alter photoreceptor number, *per se*, but may affect the ratio of rods to cones (Patel and McFarlane 2000). Increased FGF2 signaling also reduces the number of Muller glial cells and increases the number of retinal ganglion cells (Patel and McFarlane 2000). These examples highlight the fact that signaling factors have diverse effects on cell fate specification. Local signaling can profoundly influence cell types generated in culture.

Retinal transcriptional cascades. There are a large number of transcriptional regulators that are involved in retinal cell fate decisions. Several of the same genes that establish the eye field also are expressed in distinct patterns in the developing retina (Perron and Harris 1999). *Otx2*, *Pax6*, *Rx1*, and *Six3* are expressed by the stem cells at the margins of

the retina, and it has been proposed that their collective expression maintains a retinal identity in these cells (Perron et al. 1998). *Optx2* has been implicated in transforming pigmented retina into neural retina (Toy et al. 1998). Many of these genes also are implicated in regulating the proliferation of the optic vesicle and cup progenitor cells (Martinez-Morales et al. 2001; Loosli et al. 2001). For example, retinoblasts transfected with *Optx2* produce clones that are 2-fold larger that control clones, and its overexpression early in embryogenesis results in giant eyes (Zuber et al. 1999).

Furthermore, eye field genes are differentially expressed as retinal cells differentiate (Perron and Harris 1999). Ganglion and amacrine cells express *Pax6* and *Six3*; cells in the outer zone of the inner nuclear layer express *Six3*, *Rx1* and *Otx2*; and photoreceptor cells express *Rx1*. Added to this complexity is the differential expression of a large number of basic helix-loop-helix (bHLH) differentiation factors (Perron and Harris, 1999; Vetter and Brown 2001). Different combinations of these latter genes are expressed as cells transit from the purely stem cell zone at the margin, through more restricted progenitor zones and finally into their retinal layers. Photoreceptors require *NeuroD* and *Ngn2*; bipolar cells require *Ash1*, *Ash3* and *Ngn2*; amacrine cells require *NeuroD* and *Ath*3; and ganglion cells require *Ath5*. There is compelling evidence that *Pax6* directly regulates several of the bHLH genes (Marquardt et al., 2001) and that *Pax6, Six3,* and *Rx1* modulate a cell's responsiveness to the bHLH genes (Inoue et al. 2002).

Many of these genes also cross-regulate each other—when a gene manipulation eliminates one cell class, other classes are significantly enlarged. These data indicate that the transcriptional program that specifies one cell type are interrelated with programs that specify other cell types, forming transcriptional webs. This suggests that individual and evolutionary changes in the expression of a single genes, the main topic of the next section, will frequently have pleiotropic effects that lead to coordinate changes of several cell types.

Part 2: Functional and genetic analysis of individual differences

The types and numbers of cells that are generated and distributed across the retina represent an explicit, although still incompletely deciphered, summary of a species' relationship with its visual world (Østerberg 1935, Walls 1942, Hayes and Brooke 1990). These cellular parameters can be modified relatively rapidly in response to changes in selective pressure (Williams et al. 1993; Jeffery et al. 1998, 2000). Variation is by no means limited to retina, and there are even more striking quantitative differences in the size and structure of the complex quilt of visual areas that extend from occipital pole into temporal and parietal neocortex of mammals (Kaas 2003, this volume; van Essen 2003, this volume).

In this section we first consider the functional relevance of variation in numbers of neurons and photoreceptors and review what we are beginning to learn about the genetic basis of individual differences within species. As illustrated by a few specific examples as the end of this section, variation in retinal structure traces back allelic differences that affect developmental processes.

Functional perspective on cell population size

Need for large numbers. An enormous number of cells and synapses are needed to generate and interpret multiple neural representations of the visual world. The advantage of having large numbers of cells is most obvious in the vertebrate fovea (e.g., Wikler et al. 1990, Collin and Collin 1999). Cones are often packed together in a tight triangular mosaic with peak densities that reach up to 300,000/mm2 in humans (Curcio et al. 1987, 1990). Trios of photoreceptors, midget bipolar cells, and retinal ganglion cells are organized with near pixel-like precision to recapture and transmit the high spatial frequencies into the central visual system. Several remarkable developmental events help to generate high numbers in central retina (Lia et al., 1987; LaVail et al. 1991; Hendrickson 1994;Provis et al. 1998; Springer 1999), the most surprising being a shift of cones toward fovea from surrounding retina late in retinal development (Packer et al. 1990).

The need for large numbers cascades through the dorsal lateral geniculate nucleus (LGN) to the primary visual cortex. Ratios of retinal ganglion cells and projection neurons in the LGN are close to 1:1 in both rhesus monkeys and humans (Williams and Rakic 1988; Spear et al. 1996). Cell densities are about 25% higher in the posterior region that represents fovea (Ahmed and Spear 1993). Numbers increase 300- to 400-fold in visual cortex. An average of 350 million neurons are packed into area 17 of both macaques and humans (Suner and Rakic 1996; Tolhurst and Ling 1988). Cell densities may be twice as high as those in other neocortical areas (Rockel et al. 1980, Powell and Hendrickson 1981). A staggering sum of 2–3 trillion synapses are involved in the first stage cortical analysis of the visual world (O'Kusky and Colonnier 1982; Colonnier and O'Kusky 1981). Further amplifying these numbers, area 17 devotes disproportionate computational resources to the foveal representation. The central 1 mm2 of both retinas contain a total of about 150,000 cones that view the central 12 deg2 of visual space. These receptors feed signals bilaterally to 400–500 mm2 of visual cortex that contains approximately 50 million neurons and 150 billion synapses (estimate computed for both hemispheres by combining data in van Essen et al. 1984, Tolhurst and Ling 1988; O'Kusky and Colonnier 1982).

Functional correlate. A behavioral corollary of high numbers in fovea and the visual cortex is extremely high acuity, i.e., an ability to resolve spatial frequencies of 60–100 cycles per degree (Banks et al., 1991). Even more remarkable, humans can detect misalignments of short line segments finer than the grain of the photoreceptor mosaic. Detection thresholds can be as low as 10 arc seconds. This computational feat, referred to as vernier hyperacuity (Westheimer 1981; Kumar and Glaser 1993), depends in part on maintaining high spatiotemporal precision across large numbers of coactivated neurons (Kandil and Fahle 2001).

Numbers and neural noise. Impressive visual capabilities of this type depend on minimizing noise at all levels of the retinogeniculocortical system. Noise is suppressed roughly as a function of the square root of n, where n is numbers of active neurons, synapses, and synaptic vesicle fusion events (Sterling 1998). Small gains in signal-to-noise ratios may appreciably improve fitness due to increased acuity, and this in turn may have fueled steep evolutionary increases in numbers of cells in several vertebrate lineages.

For example, the prominent expansion of the peristriate visual cortex in hominids may have been driven both by the complexity of simultaneously processing multiple streams of visual information (Dacey 2000) and by selective pressure to improve signal-to-noise ratios of cortical circuits.

Even low-light vision requires large numbers of cells. Vision in low light also demands high numbers of cells, but for reasons other than visual acuity. A wide belt of the retina that usually surrounds the fovea at 10–15 degrees of eccentricity has rod densities that can rise to 500,000–850,000 cells/mm^2 in primates (Wikler and Rakic 1990, Dkhissi-Benyahya et al. 2001), cats (Williams et al. 1993), and mice (Jeon et al. 1998). Even in the far periphery of humans, rod density is still typically above 40,000 cells/mm^2 (Williams 1991).

An explanation for the high density and small size of rods is that they must be able to convert the absorption of a single photon into a distinct reduction in the transmitter release (Rodieck 1998). This requires an exceedingly slender rod outer segment in which a single photon will generate a sufficient reduction in cGMP concentration to shut cation channels. The high impedance of rods converts small fluctuations in outer segment currents into corresponding voltage fluctuations at the synaptic terminal. The great majority of the outer surface of the retina is tiled by rod outer segments to ensure efficient capture of the few photons available under low light. Their signals are averaged and integrated by AII amacrine cells, and these cells appear to define the acuity limit under low light conditions (Mills and Massey 1999), much as ganglion cells densities define the acuity limit in daylight.

Because of the inherent noise of the sporadic arrival of photons and receptor noise generated by spontaneous isomerization of rhodopsin and Poisson variance in vesicle release, detecting and interpreting objects in the dark is a challenging computational task. The consequence of these particular environmental and photochemical characteristics is that the population of rods in the human retina is incredibly high—typically in the range from 100 to 150 million (Curcio et al. 1990). Even a mouse retina has approximately 6 million rods. Having very large photoreceptor populations ensures high acuity (cones) in daylight or higher sensitivity and lowered noise levels (rods) in the dark.

Cell function and cell numbers. The functional relationship between cell number and visual system performance is not always as clear-cut as suggested by these examples. There are approximately sixty cells types in the retina and certainly just as many more in the central part of the visual system, each of which by definition makes somewhat different contributions to vision. Some cells, such as horizontal cells and the population of photosensitive retinal ganglion cells that project to hypothalamic circadian pacemaker cells, have computational roles that can best be summarized as averaging mean illuminance (Hattar et al. 2002). In the case of horizontal cells, this averaged signal provides a negative feedback to photoreceptors. Losing all horizontal cells has severe consequences, reducing retinal transduction efficiency and increasing the latency of visual evoked potentials in the cortex (Peachey et al. 1997). However, the crucial biometric parameter in this case is not numbers of channels but uniform field coverage. Small numbers of cells with extensive dendritic and axonal arbors may provide equivalent or even superior feedback compared to larger numbers of cells with smaller

fields. Cells with this sort of role will have a number:performance function that is relatively flat or even negative when numbers exceed the optimum.

Evolutionary flexibility of numbers. Given these considerations, populations of neurons in evolving species will ratchet up or down in numbers at somewhat idiosyncratic rates that depend on their functional contributions to visual performance. For example, populations of rods are higher in domestic cats than in ancestral wildcats (Williams et al. 1993), and populations of retinal ganglion cells are lower in domestic cats and dogs than in wildcats and wolves (Peichl 1992; Williams et al. 1993); in addition, both domestic species are likely to have lower acuity than their wild peers. Numbers of neurons will reach asymptotes at which further changes are blunted by countervailing selective pressure, such as the metabolic load associated with increased population size (Franco et al. 2000). The final cell number is a compromise between the marginal utility of adding yet more cells and the metabolic costs that these extra neurons inevitably incur. An interesting consequence of reaching a functional asymptote is that cell populations within a species will typically be maintained at levels around which significant numerical deviation is surprisingly well tolerated by individual animals (see the list below). Near the asymptote the relation between number and function is nearly flat, and visual system performance will be robust even in the face of surprisingly large deviations in numbers and ratios of cells that may be introduced by rare alleles and mutations, population admixture, developmental noise, environmental perturbation, or disease.

Variation within species and genotype. Ranges of normal variation in absolute numbers of cells and in ratios of cells within a species are often large. There is usually no obvious functional effect. The estimates of variation that are listed below come from small samples and these examples therefore are likely to underestimate the actual range of variation. In most cases, the examples illustrate variation among individuals.

1. Foveal cone densities vary at least 3-fold among humans without retinal disease (Curcio et al. 1990; $n = 8$).

2. Ratios of L and M cones (red and green) in human central retina can range from 1:1 to 3.8:1 without evident (or with only subtle) differences in the categorization of colors (Roorda and Williams 1999), $n = 2$).

3. Even within a single retina, the ratio of rods and cones can vary two-fold. In one case, adjacent large fields in the periphery of a normal human retina had rod:cones ratios of 1:2 and 2:1 (Williams 1991).

4. Two-fold differences in horizontal cell number and density are common among normal inbred strains of mice (Williams et al. 1998a). A single strain of inbred mouse does not provide a good index of a typical ratio of cell types (see Williams et al. 1998b). For example, the ratio of ganglion cells to horizontal cells in mice varies from 3.2:1 to 6.7:1.

5. The population of retinal ganglion cells—the information bottleneck of the entire visual system—varies from 1,000,000 to 1,600,000 in humans and macaques (Rakic and Riley 1983, Provis et al. 1985; Spear et al. 1996). This population varies from 45,000 to 75,000 among strains of mice (Williams et al. 1996).

6. The population of projection neurons in the dorsal lateral geniculate varies as much as two-fold within cat, mouse, and macaque (Williams and Rakic 1988, Ahmad and Spear 1993, n <10 cat and macaque, n = 100 in mouse). For example in mouse, the population of projection neurons varies from 12,000 in CE/J to 22,000 in AKR/J (Kulkarni et al. 2000).

7. The surface area and cell population of primary visual cortex in both humans and macaques varies two- or even three-fold (van Essen et al. 1984; Suner and Rakic 1996; Gillisen and Zilles 1996; Leuba and Kraftsik 1994; n of up to 20).

8. Ocular dominance column numbers along the vertical meridian range from 101 to 154 in macaques. Width of columns ranges from 400 to nearly 700 µm (Horton and Hocking 1996; n = 12 hemispheres).

The paradox of high variation. The magnitude of this variation may be puzzling, particularly in species that are highly dependent on vision. Stabilizing selection, a process that normally trims away the tails of a distribution of phenotypes, would normally limit the range of variation that is tolerated in an interbreeding population. But the examples above provide ample demonstration that wide variation is well tolerated even in species such as macaques and humans that are utterly dependent on vision. The apparent paradox is resolved if we come back to the point that the visual system is over-engineered and that there is much functional and developmental redundancy built into the retinas of most individuals. This also means that directional selection has plenty of variation with which to work on an evolutionary scale. Selection is highly effective in changing the cellular demographics of the retina and other parts of the visual system. Rapid changes in cell number have occurred in at least two carnivore lineages—wolves and wildcats—over an interval of less than 20,000 years (Peichl 1992; Williams et al. 1993). In cats, the population of retinal ganglion cells has eroded from about 240,000 in wildcats to 160,000 in domestic cats. Even more extreme changes have been uncovered in response to rapid changes in habitat, for example, isolation in lightless cave systems in which the eyes regress due to the accumulation of deleterious mutations in genes critical at several stages of eye development (Jeffery and Martasian 1998).

Genetic basis of individual difference in retinal structure

Genetic polymorphism within species. The striking differences in retinal architecture of mouse and human arose from natural genetic variation generated and selected within single species. Roughly one-fifth of genes in most species have functional polymorphisms that produce changes in the electrophoretic mobility of the corresponding proteins. This reservoir of genetic variation often translates to corresponding high heritability estimates for quantitative retinal traits (Williams 2000; Zhou and Williams 1999). For example, variation in retinal ganglion cell number in mice has a heritability above 80% (Williams et al. 1996), demonstrating strong genetic modulation of this population under typical laboratory conditions. The goal of a new field of genetics called complex trait analysis is to convert these bland estimates of heritability into the much more interesting and informative gene variants. These gene variants or polymorphisms can in turn be used to explore the genetic control of retinal development.

Mendelian and quantitative genetics. Mendelian methods of gene analysis with which most readers will be familiar, assume that single gene variants—usually rare alleles or mutations—generate essentially all interesting differences. For example, mutations of the tyrosinase gene (albinism) causes a 35% reduction in the fraction of retinal ganglion cells with ipsilateral projections in mice regardless of genetic background (Rice et al. 1995).

In contrast to Mendelian genetics, complex trait analysis begins with the assumption that differences in cell populations are generated by the combined effects of a large number of polymorphic genes scattered across the genome. The goal is to map this set of genes to precisely delimited chromosomal locations and then proceed to identify these genes and the molecular pathways of which they are part. The types of genes that are part of these multigenic systems are known as quantitative trait loci or QTLs. Overwhelming technical difficulties prevented QTLs from being mapped in vertebrates even a decade ago. However, with the introduction of efficient PCR-based methods to genotype animals and sophisticated statistical programs to discover associations between gene variants and phenotypes, the prospects for QTL mapping are now greatly improved. It is possible to target virtually any heritable quantitative trait for what is called genetic dissection. This is particularly the case if one can exploit strains of inbred mice.

Use of isogenic lines. An inbred strain is essentially an isogenic clone of fully homozygous animals that are able to reproduce sexually. They are a remarkable tool with which it is possible to resample the same genetic individual at different stages and after different treatments. They can also be used to obtain reliable quantitative estimates of traits that have high noise that is caused either by technical error or by stochastic developmental variation. If two or more inbred lines differ substantially for a set of retinal traits under a common environment, then the difference traces back to genetic differences between those lines. These characteristics have been exploited to map and characterize novel gene loci that modulate cell populations in the retina.

The population of retinal ganglion cells has been counted in over 1000 mice belonging to 60 isogenic lines. Numbers range from 45,000 in strain CAST/Ei to 75,000 in BXD32 (Williams et al. 1996). While individual values have a normal distribution, when cases are pooled by strain the histogram has a characteristic multimodal structure in which the different modes represent different combinations of genotypes at several QTLs. The most prominent pair of modes is present in neonatal mice before ganglion cells have been eliminated by apoptosis (Strom and Williams 1998). This indicates that the QTL responsible for these modes, the *Nnc1* locus, has a modulatory effect on the proliferation or differentiation of retinal ganglion cells.

Mapping neurogenic genes. Crosses between strains with high and low ganglion cell number have been used to map *Nnc1* and three other QTLs responsible for much of the variation in ganglion cell number (Williams et al. 1998b, Strom 1999). The gene mapping method can be distilled to statistical tests of association between differences in cell number and differences in genotype (genotypes are often scored in a numerical format: AA = 1, Aa = 0, aa = −1). For example, the correlation between ganglion cell number and genotypes at *Nnc1* reaches a peak of 0.72 on chromosome 11. The other three QTLs that modulate ganglion cell number map to Chr 7 at 65 cM (*Nnc2*, LOD score of 5.9), Chr 1 at 82 cM (*Nnc3*, LOD of 9.3), and Chr 16 at 42 cM (*Nnc4*, LOD of 6.0).

Nnc1 has the largest effect, and this QTL maps to a 6 million base pair stretch of DNA on chromosome 11 between *Hoxb* and *Krt1*. This region contains two obvious candidate genes—the retinoic acid alpha receptor and the thyroid hormone alpha receptor. The addition of exogenous retinoic acid to tissue cultures increases rod production at the expense of amacrine cells (Kelley et al. 1994). However, knockouts of the retinoic acid receptor have no detectible effect on retinal ganglion number; a surprising result given the high expression of this gene in ganglion cells from very early stages (Zhou et al., 2001). In contrast, inactivation of the thyroid hormone alpha receptor neatly reproduces a 15% predicted difference in cell number. This result suggests that the QTL on Chr 11 corresponds to a polymorphism of the thyroid hormone receptor gene (Strom 1999), but this idea has not yet been confirmed by sequence comparison. However, the hypothesis is attractive because thyroid hormone has long been known to control retinal ganglion cell proliferation during *Xenopus* metamorphosis—a stage in this species' development when a population of ipsilaterally projecting ganglion cells is first generated (Hoskin 1985). The thyroid receptor is a transcription factor that often pairs with a retinoid X receptor to control gene expression. It may not be just coincidental that the QTL on Chr 1 that modulates ganglion cell number overlaps the position of the *Rxrg* gene (Strom 1999).

Conclusion

It is already apparent that many developmental processes are intertwined in such a way as to maintain general conformity in retinal structure and cellular content while still permitting allow substantial latitude for individual variation. A growing number of molecules and processes are now known to affect the diversity and numbers of retinal cells types generated in multiple steps during retinogenesis (Harris 1997; Cepko 1999; Livesey and Cepko 2001; Vetter and Moore 2001; Vetter and Brown 2001). Experimental tests to sort out their relative contributions also demonstrate that there are numerous stages at which the final numbers and relative proportions of the different retinal phenotypes can be changed. Understanding this process of differentiation at a quantitative level will require an analysis of the transcriptome and proteome of the lineages of the major cell types, and an understanding of the processes that occur during the very early embryonic stages to bias and then select the retinal stem cells. Although we are still far from being able to navigate through the molecular labyrinths that generate different retinal cell types in appropriate numbers, the pace of research is now so rapid that it is hard not to be optimistic.

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LEGENDS

Figure 1: Early steps of retinogenesis. Retinal development begins with several steps that set aside embryonic precursors. First, a subset of embryonic cells (green) becomes competent to contribute to the retina. The blue-hatched cell is inhibited from forming retina. Next, a smaller subset (yellow) is biased to become the retina-forming cells. Next, a specified retinal stem cell population (red) emerges from the descendants of the biased embryonic cells (yellow) to form the eye field in the anterior neural plate (green). Finally, during neural tube morphogenesis this specified population is segregated into three major compartments: neural retina (red), pigmented retina (black) and optic stalk (blue).

Figure 2: Retina competent and biased blastomeres in *Xenopus laevis* embryos.
(Left): Animal pole view of a 32-cell embryo showing the major (orange) and minor (yellow) blastomeres that contribute to the retina. The numbers within each cell indicate the percentage of retinal cells that derive on average from each blastomere.
(Right): Side view of a 32-cell embryo showing the retina-forming blastomeres (yellow), the blastomeres that do not make retina but are competent to do so (green) and the vegetal blastomeres that are inhibited by maternal factors from making retina (blue).
Data are from Huang and Moody (1993). An, animal pole: D, dorsal; V, ventral; Veg, vegetal pole.