Review

Cellular pattern formation in the retina: retinal regeneration as a model system

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Like many structures in the central nervous system, the neural retina is highly organized at the cellular level. Examples of this cellular organization include the laminar profile of the vertebrate retina, the hexagonal array of ommatidia in the retinas of insects, and non-random two-dimensional patterns of specific vertebrate retinal neurons. These organized cellular ensembles are taxonomically robust, and their importance in visual processing is, although typically not well understood, virtually axiomatic. The presence of non-random cellular patterns in the retina also begs questions concerning the spatial nature of the patterns, and the underlying mechanisms that coordinate their assembly during retinal development and growth. What are the spatial characteristics of the non-random cellular patterns? What molecular signaling schemes might account for their assembly? What are good model systems for investigating these issues? In this review we attempt to provide some preliminary answers to these questions. We present recent advances in our understanding of cellular patterns in the vertebrate retina and the mechanisms that underlie their assembly, the ability of adult anamniote retinas to regenerate following injury, and how these seemingly disparate topics can be successfully merged into an effort to better understand both processes. We combine insights from retinal assembly mechanisms in Drosophila with empirical, quantitative, and theoretical investigations in vertebrates, to propose an inclusive model for retinal cell patterning.

CELLULAR PATTERNS IN THE RETINA

The most commonly recognized element of non-random cellular organization in the vertebrate retina is its laminar profile of three nuclear and two plexiform layers. The somata and processes of the six major classes of retinal cells (photoreceptors, Müller glia, and horizontal, bipolar, amacrine, and ganglion cells) reside at stereotypical positions within this laminar structure (Figure 1A). The molecular and cellular mechanisms that regulate the formation of this laminar structure have received considerable experimental attention recently, particularly in studies that involve mutant strains of zebrafish (e.g., [1-3]), but discussion of this topic is beyond the scope of this paper.

A significant, although less well known, component of the retina’s non-random cellular organization resides within the tangential plane. The mammalian fovea is a classic example of such a non-random structure (reviewed by [4]), but a different aspect of non-random cellular organization is present in perhaps all vertebrate retinas, even those that lack a fovea. Recognized since at least the middle of the nineteenth century (Figure 1B; [5]), this cellular organization is characterized by two-dimensional distributions of like-type cells that are non-random. Perhaps the most graphic examples of non-random cellular patterns within the retina’s tangential plane are the highly regular mosaics of cone photoreceptors that are typical of teleost fish. Within these crystalline-like mosaics, specific cone types are located at predictable positions relative to each other cone type (e.g., [6-10]; see Figure 1C). Non-random patterns of cones have also been observed in primates [11,12], and non-random arrays of inner retinal neurons have also been frequently observed in a number of vertebrate taxa [13-27]. A common feature of all of these non-random patterns is local ‘anti-clustering’; that is, for each soma of a certain type ‘A’, there will be a surrounding region of two-dimensional space within which the probability of encountering another soma of type ‘A’ is extremely low (Figure 1B,C; reviewed by [26]).

DEVELOPMENT OF RETINAL CELL MOSAICS: INSECTS AND VERTEBRATES

The ubiquity of these non-random cellular patterns, ubiquitous across not only disparate retinal strata but also across disparate species, suggests a physiological and/or developmental significance that is more than epiphenomenal. Although the physiological significance of these patterns is not completely understood, the presence of non-random cellular patterns indicates that spatial organizer mechanism(s) must operate during retinal development and growth.

Much of our knowledge about the mechanisms that regulate cellular pattern formation in the retina has come from studies of invertebrate eye development, particularly those involving the Drosophila eye (reviewed by [28-32]). These studies have been successful at identifying multiple molecular agents, genetic pathways, and cell-cell interactions that are directly
involved in neurogenesis and pattern formation during ocular
development. For example, patterning of ommatidia in the
*Drosophila* imaginal disc involves the progression of a linear
wave of mitoses called the morphogenetic furrow. Furrow pro-
gression requires a diffusible signal, the secreted protein
Hedgehog, from previously-differentiated photoreceptors. This
signal propels the morphogenetic furrow by inducing a linear
array of cells to undergo synchronized mitoses and express
the transcription factor *atonal* (reviewed by [32]). *Atonal*
expression then becomes restricted to evenly-spaced clusters of
cells by the action of diffusible, inhibitory signals that are re-
leased by a slightly older cohort of retinal cells, and is then
further restricted to evenly-spaced individual cells by the in-
hibitory action of the Notch-Delta system ([33]; Figure 2).
This molecular signaling system results in a precise pattern of
*atonal*-expressing cells that are fated to become R8 photore-
ceptors, which in turn serve as the “founder” differentiated
element of the regularly spaced ommatidia. The R8 photore-
ceptors initiate a cascade of position-dependent, cell-cell in-
teractions that induce surrounding cells to adopt specific, non-

Figure 1. Cellular organization of the vertebrate retina. A: Laminar arrangement of vertebrate retinal cells; schematic obtained from Webvision,
The Organization of the Vertebrate Retina, with permission. B: Schematic of the cone photoreceptor mosaic of Perca fluviatilis (European
perch) from p. 329 of Hannover [5]. The cylindrical profiles represent double cones (“Zwillingzapfen”), and the circular profiles represent
single cones. We believe this to be the first published description and illustration of a teleost cone mosaic. C,D: Cone mosaic of zebrafish. C:
A fragment of whole retina hybridized with cRNA probes corresponding to the opsin messages for S (“blue”) cones (bl, red fluorescence) and
UV cones (uv, green fluorescence). D: A schematic of the zebrafish cone mosaic, illustrating the spatial relationship of the different spectral
cone types. Purple, UV cones; Blue, “blue” cones; Green, “green” cones; Red, “red” cones. The juxtaposed “red” and “green” cones constitute
the double cone morphology.
R8 photoreceptor cell fates (Figure 2). The best-described example of these R8-mediated interactions is the induction of the R7 cell via interaction of the membrane proteins Sevenless and Boss (reviewed by [34]). Cellular pattern formation in the *Drosophila* eye thus involves a complex array of signaling systems, the components of which include agents that promote mitosis, inhibit differentiation, or promote differentiation.

In contrast, relatively little is known about the cellular and molecular mechanisms that control cellular pattern formation in the vertebrate retina. Recent studies have confirmed that vertebrate homologs of *Drosophila* retinal patterning genes are expressed during retinal development. For example, *Hedgehog* and *atonal* homologs have each been shown to participate in driving a wave of retinal development [35-38], although direct interactions between these two genes have yet to be reported. Additionally, the Notch-Delta signaling system may also contribute to neurogenesis in the vertebrate retina by regulating the choice to differentiate or remain proliferative [39,40], or in neuronal vs. glial fate choice [41,42]. How these specific genetic pathways contribute to specific aspects of cellular pattern formation, however, has not been studied.

As an alternative to characterizing homologous patterning genes, other studies have focused upon descriptive similarities in retinal development between *Drosophila* and vertebrates. Raymond [43] proposed that the regular array of cone photoreceptors in fish arises from a series of position-dependent, inductive interactions that follow the specification of a “founder” photoreceptor which, like the R8 photoreceptor of *Drosophila*, differentiates rapidly and then sets up the regular spacing of the subsequent photoreceptor array. The presence of a stereotyped sequence of opsin expression during retinal development seems consistent with this hypothesis (goldfish: [44,45]; zebrafish: [46,47]). For example, rod photoreceptors differentiate first (“differentiation” is here operationally defined as the expression of a specific opsin), followed sequentially by red-, green-, blue-, and ultraviolet-sensitive cones. Furthermore, the sequence of cone differentiation predicts the position of each cone type within the mature mosaic because it is correlated with increasing distance from the first cone type (red-sensitive) to differentiate. Because the rod photoreceptor differentiates prior to any cone photoreceptors, and because it has a qualitatively regular array in the early retina, it has been proposed as a potential “founder” photoreceptor for the teleost cone mosaic ([44,45]; see discussion by [27]). Although a “founder” role for rod photoreceptors has since been discounted ([48]; see also [49]), position-dependent “founder” mechanisms for the regulation of differentiation (or perhaps determination) of cone photoreceptors in teleosts remain possible.

Interestingly, descriptive studies in other taxa have revealed alternative sequences of opsin expression. However, consistent among all these studies is a non-random temporal characteristic: the different cone types differentiate asynchronously [44-47,49-51]. Another striking consistency across species is that for each photoreceptor type, a non-random pattern is evident at the initial time at which each cell type can be identified, consistent with a role for position-dependent mechanisms in cellular differentiation. This feature has been noted for inner retinal neurons as well. For example, McCabe et al. [21] have found that from their first manifestation as a distinct, differentiated cell type, retinal ganglion cells are arrayed in non-random, two-dimensional patterns. This feature has also been observed for several distinct sub-types of inner retinal neuron in the zebrafish (Cameron and Carney, unpub. Obs.). These studies indicate that the mechanisms that spatially regulate cellular pattern formation operate at relatively early time points during the cellular differentiation process, and therefore may be distinct from later events such as cellular movements [52-55] or programmed cell death (reviewed by [56,57]).

The temporal interval between photoreceptor birth (terminal mitosis) and opsin expression can, however, be quite long [45,48]. This observation admits the possibility for patterning mechanisms, such as cell movement, that may operate during this interval, although there is yet no evidence that this occurs outside the fovea. At minimum, this also argues against opsin proteins themselves functioning as an inter-cellular signaling mechanism to regulate early events in photoreceptor development [48], and it also suggests that relying upon opsin expression as an indicator of photoreceptor differentiation may mask the detection of early molecular events that are critical components of photoreceptor pattern formation. This interpretation is supported by the results of two recent studies of *Drosophila* eye development. In the first report, expression of the Rh5 opsin in R8 photoreceptors was induced by Rh3-expressing R7 cells, which differentiate after the R8 cells (see above; [58]). In the second, which involved *salml/salr* mutants, a lack of expression of the R8 photoreceptor opsin (Rh5 or Rh6) did not compromise the subsequent R8-mediated induction of other photoreceptor types, nor did it compromise other features specific to the R8 cell phenotype (axonal projection to the medulla) [59].

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**Figure 2. Drosophila ommatidial development.** 1. Morphogenetic furrow (purple) is propagated from posterior to anterior (bottom to top) across the eye disc by *Hedgehog* (black arrows), expressed by developing photoreceptors (gray). 2. *atonal* expression (black) becomes restricted to R8 photoreceptor by inhibitory interactions (red arrows). 3. Progressive recruitment of non-R8 photoreceptors (blue) by inductive/permitive interactions (green arrows).
MECHANISMS OF CELLULAR PATTERN FORMATION IN THE RETINA: INSIGHTS FROM QUANTITATIVE PATTERN ANALYSES

An additional approach that has yielded considerable insight into the mechanisms that regulate cellular pattern formation has been the application of quantitative pattern analyses. Two such analyses are nearest-neighbor distance (NND; [17]) and the density recovery profile (DRP; [60]). Both NND and DRP analysis are correlative, analytical tools that evaluate, for a population of objects, the spatial distance between said objects; in most applications that involve the retina these objects are defined as somata (or inner segments in the case of photoreceptors), and the analyses are typically restricted to two-dimensional space. NND evaluates the distance of each soma to its nearest neighboring soma of like- (auto-correlation) or unlike- (cross-correlation) cell-type. The DRP, which can also operate in auto- or cross-correlation modes, evaluates the distance of each soma to most of the other somata within the population, from which the spatial density of the surrounding somata is “recovered” with increasing annular distance away from the reference cell. A feature of both analyses is that they can provide a statistical foundation for evaluating relatively local (i.e., short-range) characteristics of two-dimensional patterns.

Figure 3. Pattern analysis of serotonin-positive cells in the inner retina of an adult zebrafish. Analysis of the pattern of serotonin-positive cells in the inner retina of an adult zebrafish, illustrating pattern characteristics that are common for inner retinal cells of various types. A, Nearest neighbor distance (NND) distribution, as a function of the fraction of cells within the analyzed sample. Note how the distribution is well estimated by a normal (Gaussian) function (red line), consistent with a non-random distribution of points. Conformity ratio analysis of the NND distribution [17] also reveals the two-dimensional pattern to be non-random. B, Density recovery profile (DRP) [60] analysis of the same pattern of serotonin-positive cells. Each serotonin-positive soma (abscissa value 0) is surrounded by a two-dimensional area that is devoid of other serotonin-positive somata (i.e., the density values equal zero below 30 µm). The density of the sample (black horizontal line) is not “recovered” until an annular area approximately 60 µm away from each soma is sampled. The recovered density thereafter approximates the density of the entire sample.

Figure 4. Hypothetical signaling schemes for regulating cellular pattern formation in the growing fish retina. Top: Differentiated inner retinal cells of a particular type (“cyan”) inhibit the differentiation of like-type cells via a signaling mechanism that is modeled as a decaying exponential (arbitrary ordinate scale). Unlike cell types are not directly affected. Ongoing modeling work has suggested that this relatively simple signaling scheme provides good estimates of the observed patterns for several distinct inner retinal cell types (unpublished data and [70]), and thus may represent a general mechanism for the regulation of cellular patterns in the inner retina. Bottom: At the level of cone photoreceptors, ongoing modeling work (Cameron and Carney, in preparation) suggests that signaling mechanisms that affect both like- and unlike-cell types are required for the establishment of square-shaped cone patterns similar to those illustrated in Figure 1 above. In the illustrated case, the “red” cones inhibit their own differentiation, but promote the differentiation of the “blue” cone type; the “blue” cones exert the opposite influence.
When applied to cellular patterns in the vertebrate retina, these analyses have revealed that the majority of cell types are arrayed in non-random patterns. Almost invariably, auto-correlation NND analysis of any vertebrate retinal cell class, be it photoreceptors or inner retinal cells, reveals values that are normally distributed, and mean values and mean/standard deviation ratios that are significantly greater than those expected for a random distribution (e.g., [14,17,22,61,62]; Figure 3A,B). Auto-correlation DRP analysis of the same cell types typically reveals a zone around each soma, termed the “effective radius,” that is considerably larger than the soma diameter and within which the probability of encountering another soma of like-type is extremely low ([22,24,60,63-65]; Figure 3A,B). Together, these analyses indicate that most cellular patterns in the vertebrate retina share a common feature: local anti-clustering. This pattern feature suggests that the mechanisms that regulate cellular pattern formation during development are dominated by relatively local signals that inhibit the presence of nearby like-type cells. This type of signaling scheme is reminiscent of known regulators of cellular differentiation, such as the Notch-Delta system (reviewed by [66]). Computational modeling studies of photoreceptor pattern formation in the teleost retina have also suggested the operation of inhibitory signaling schemes [67,68].

When applied specifically to cells of the inner retina, NND and DRP analysis have revealed that the patterns of like-cells are also anti-clustered [22,24,60,69], suggesting that cellular pattern formation within all layers of the retina is dominated, perhaps exclusively so, by relatively local signaling mechanisms [55]. These analyses have also indicated that the patterns of unlike-cell types in the inner retina are independent [22,24]. For example, the position of a serotonin-positive amacrine cell soma provides no predictive information about the location of the somata of any other, unlike-amacrine cell type, such as those amacrine cells that are immuno-positive for somatostatin. These results suggest the operation of multiple spatial pattern regulators, each of which is “cell-type autonomous.” Although they cannot be ruled out, inductive signaling schemes that involve communication between unlike differentiated cell-types may not be required to account for the observed cellular patterns in the inner retina. Ongoing computational modeling studies on the mechanisms of cellular pattern formation in the zebrafish retina have supported these interpretations [70].

Conversely, when applied to the patterns of cone photoreceptors in teleost fish, the same quantitative analyses reveal positive spatial correlation between like- and unlike-cell types [65]. This observation admits the possibility that pattern formation at the level of cone photoreceptors involves the activity of signaling mechanisms that are not cell-type autonomous. That is, inductive signaling events that regulate cone pattern formation (and perhaps also differentiation) may include direct interactions between different cone types. Preliminary computational investigations of cone pattern formation have also supported the idea of cross-cone-type signaling during pattern formation, and have additionally suggested that the formation of regular, square-like mosaics of cone photoreceptors in teleost fish may require the operation of inter-cone-type signaling mechanisms [70].

Therefore, quantitative and computational analyses of inner retinal cell patterns support the primary involvement of a single signaling mechanism that is cell type-autonomous and inhibitory. In contrast, analyses of cone photoreceptor mosaics support the involvement of a dual signaling scheme: an inhibitory signaling system that prevents like-cell types from occupying nearby space, and an inductive and/or permissive signaling system that enables unlike-cell types to occupy nearby space (Figure 4). This dual system is remarkably similar to the known signals that regulate formation of the *Drosophila* ommatidial mosaic, as presented earlier: a set of inhibitory signals that progressively restrict R8 cell fate to form an evenly-spaced array of “founder” cells, and a set of inductive/permissive signals that instruct cells adjacent to R8 photoreceptors to adopt non-R8 cell fates.

Where, and from which cells, do these signals arise in the vertebrate retina? At what developmental time points do they operate? What happens when these signaling systems are disrupted? Preliminary answers to these questions come from a surprising source: the study of retinal cell patterns in regenerated retina. In the following sections we will briefly review the process of retinal regeneration in teleosts, present quantitative analysis of cellular patterns in regenerated retina, and then discuss how these observations have provided insights into the mechanisms that underlie cellular pattern formation.

**RETINAL REGENERATION IN ADULT VERTEBRATES**

It has been known for many decades that the retinas of adult anamniotes can replace neurons that are lost due to injury [71-73]. When a small piece of the retina is surgically removed from an adult fish, for example, a proliferative blastema forms along the lesion edge, from which new, replacement neurons are produced [74]. Similarly, when the fish retina is damaged subsequent to exposure to the cytotoxic agent ouabain, regenerated retina is produced from spatially discrete neurogenic foci scattered throughout the damaged retina [65,75,76]. In both situations, proliferative cells within the neural retina that survive the injury are believed to repopulate the damaged retina with new neurons (reviewed by [77]).

Although the identity of the proliferative cell type(s) that produce regenerated retina is not known with certainty, the teleost retina contains several candidate cell types that could perform this “stem cell” function. These cells include: rod precursors, which reside within the outer nuclear layer and produce rod photoreceptors throughout the life of the fish [78,79]; proliferative inner nuclear cells (PINCs), which reside within the inner nuclear layer and are a likely source of rod precursors [80-82]; and stem cells that reside within a neuroepithelium at the ora, termed the circumferential germinal zone (CGZ), from whence retinal neurons are produced and appositionally added to the retina throughout the life of the fish, in a manner reminiscent of the growth of a tree trunk [83-88]. Following retinal damage there is an increase in proliferation of rod precursors, PINCs, and CGZ cells [74,76,77],
indicating that all of these cell types are responsive to injury in a manner consistent with a potential role in neurogenesis. A recent report has shown that following focal laser lesions to the photoreceptor layer, a cellular source of new neurons resides in the INL [89]. In contrast to amphibians (reviewed by [90-92]), transdifferentiation of RPE has been discounted as a regenerative mechanism in teleosts [93].

Neuroregenerative potential may also reside within the retinas of amniotes. A recent report indicated that Müller glia in the chicken can respond to retinal injury by dedifferentiating, proliferating, and redifferentiating into some (but perhaps not all) retinal cell types [94]. Additionally, evidence is mounting that mammalian retinas possess cellular machinery that may support neuronal regeneration. In murines, putative retinal stem cells have been isolated from the ciliary margin [95,96] and the retina [97], and although preliminary, these reports are provocative because they suggest a potential for damaged human retinas to recruit new retinal tissue from an endogenous cellular source(s).

RETINAL REGENERATION: RECAPITULATION OF RETINAL DEVELOPMENT?

The retinal regeneration literature is replete with examples of how remarkably similar this process is to retinal development (reviewed by [77]). For example, the proliferative and ultrastructural profile of the regenerative blastema strongly resembles that of the developmental proliferative neuroepithelium, as well as the CGZ. At the molecular level the regenerative blastema expresses genes that are involved in embryonic retinal neurogenesis, including vsx-1 (a teleost homolog of chx10; [98]), pax6 [99], and Notch [100]. Preliminary evidence from studies using quantitative RT-PCR and in situ hybridization techniques [101] also indicate that retinal injury induces an increase in the expression of several genes implicated in retinal development, including pax6, vsx-1, rx3 [102], and neuroD [103]. Although these observations suggest that retinal regeneration may represent a re-deployment of a retinal developmental program, there are several aspects and outcomes of the regenerative process that seem to argue against this simple notion. For example, although proliferation of cells at the CGZ is enhanced by insulin-like growth factors, a similar effect is not observed at the regenerative blastema [85].

RETINAL REGENERATION: CELLULAR PATTERNS AND PATTERN FORMATION

Cook and Chalupa [27] reviewed cellular mosaics in the vertebrate retina, and predicted that studies of injured retina would lead to insights into the mechanisms that regulate retinal cell patterning in the native, uninjured retina. This prediction is being borne out by recent empirical and computational studies of cellular pattern formation during retinal regeneration (e.g., [22,65]).

The morphology and spatial patterns of neurons across the regenerated retina display significant differences as compared to native retina (Figure 5; [22,65,83,104-106]). This feature has been observed in all models of teleost retinal regeneration, with the patterning errors being particularly pronounced at the level of cone photoreceptors [65]. Abnormal cone morphologies, such as triple cones, have been observed in regenerated retina [83,107], and the normal densities and

Figure 5. Unusual cone patterns and phenotypes in regenerated retina. A: Cone mosaic of regenerated zebrafish retina, following a surgical lesion. Retina was hybridized with cRNA probes corresponding to blue cone opsin (bl, red fluorescence) and UV cone opsin (uv, green fluorescence). B: A triple cone, an anomalous cone morphology, in the regenerated retina of a sunfish [83]. Asterisks, the outer segments of each triple cone member; A, accessory outer segments; arrows, calical processes. The black profiles are melanin granules within projections that arise from the pigmented epithelium. Anomalous, multiple-ordered cone morphologies of this type are common in the regenerated retina of teleosts. Panel B is reproduced with permission from: Cameron DA, Easter SS Jr. Cone photoreceptor regeneration in adult fish retina: phenotypic determination and mosaic pattern formation. J Neurosci 1995; 15:2255-71. Copyright 1995, Society for Neuroscience.
ratios of cone subtypes are not recapitulated [65, 83, 107]. To date, virtually all of the inner retinal cell types examined in regenerated zebrafish retina are arrayed in patterns that are statistically less regular than their counterparts in native retina. However, although tending towards randomness, these patterns can still meet statistical criteria [17] for a non-random cellular arrangement. The planimetric density of each inner retinal cell type within the regenerated patch, however, exceeds the corresponding density in native retina [22]. Together, these observations indicate that at least some of the mechanisms that lead to the development of regular retinal cell mosaics are either not reinitiated, or are somehow significantly compromised, during regeneration.

The cellular patterns of cones and inner retinal neurons have also been quantitatively analyzed in goldfish retina that has regenerated following a chemical lesion [65]. Previous work suggested that following a chemical lesion new retina arises from two cellular sources that are spatially distinct [76]. Regenerated retina appears centrally as surviving proliferative cells intrinsic to the neural retina (e.g., PINCs) become pluripotent and produce all retinal cell classes. Additionally, new retina arises from surviving proliferative cells at the CGZ, as part of the ongoing retinal growth process. Somewhat surprisingly, both of these regions possess cone patterns that dif-

Figure 6. Unusual cone patterns and ratios in goldfish retina following a ouabain-induced lesion. A: Cone mosaic of native goldfish retina. Retina was hybridized with cRNA probes corresponding to blue cone opsin (bl, dark color product) and UV cone opsin (uv, red color product). B: Cone mosaic of peripheral goldfish retina, which had been generated after destruction of the retina by ouabain. C: Central region of the same retina, which had regenerated from stem cells that survived the toxin. D: Analysis of blue cone pattern in native (top row), peripheral (middle row) and regenerated (bottom row) goldfish retina following intraocular injection of ouabain. First column contains cartoons of the 2-dimensional pattern of cones, second column contains DRP analytical results, third column contains results from quadrat analysis, and fourth column contains NND histograms. Figure adapted from Stenkamp et al. [65]. Modified and reproduced with permission from: Stenkamp DL, Powers MK, Carney LH, Cameron DA. Evidence for two distinct mechanisms of neurogenesis and cellular pattern formation in regenerated goldfish retinas. Journal of Comparative Neurology 2001; 431:363-81. Copyright 2001, John Wiley and Sons, Inc.
fer significantly from the regular cone mosaics of native retina, and they also differ significantly from each other ([65]; Figure 6). In regenerated regions of lesioned retina, NND analysis reveals cone patterns that are not significantly different from those expected for a random pattern, and DRP analysis reveals somewhat erratic cellular patterns that possess little indication of short- nor long-range order, nor of anti-cluster-
ing tendencies.

An additional quantitative tool, quadrat analysis, was also applied in this study [108-111]. Quadrat analysis provides a statistical evaluation of whether a two-dimensional pattern of objects is regular, clumped (aggregated), or neither regular nor clumped (for example, random). This analysis is not based upon the local distances between objects, but rather upon broader pattern attributes. In brief, the area of interest is subdivided into a series of grids of contiguous equivalent boxes (quadrats), the number of objects within each box is counted for each grid, and for each grid a $\chi^2$ statistic is used to evaluate the variability of object counts. Quadrat analysis indicated that cone patterns in regenerated retina following a ouabain lesion are in fact significantly different from random, and that the non-random patterns are due to an abnormal, clumpy distribution of each cone type. Interestingly, the new retina in peripheral regions of retinas exposed to ouabain has cellular patterns similar to those observed in regenerated retina after a surgical lesion. The densities of all cone types are elevated, and NND analysis indicates that each cone pattern, although tending toward randomness, still meets the statistical criterion [17] for a non-random pattern. DRP analysis reveals compromised anti-clustering tendencies, and an absence of any overt long-range pattern. The results from quadrat analysis, however, differ with those of NND analysis in indicating that cone distributions are not significantly different from a random pattern. Because quadrat analysis emphasizes pattern characteristics over relatively large spatial extents—whereas NND analysis emphasizes primarily local characteristics—these data indicate that long-range aspects of cone patterns are more severely compromised than local aspects. Finally, these peripheral regions contain both an absolute and relative excess of blue cones (as compared to other cone types), suggesting that abnormalities in cone pattern formation can be accompanied, or perhaps driven by, errors in cone fate determination.

CELLULAR PATTERN FORMATION IN THE VERTEBRATE RETINA: RETINAL REGENERATION AS A MODEL SYSTEM

How can these data contribute to our knowledge of cellular pattern formation in the vertebrate retina? Consider first that in all cases of retinal regeneration examined, cone patterns are more severely disrupted than the patterns of inner retinal neurons [22,65]. One interpretation of this observation is that the establishment of cone patterns is either more sensitive to, or more readily compromised by, changes in the retinal milieu. Consistent with this interpretation are the empirical and computational studies that suggest cell type-autonomous signaling schemes for regulating inner retinal pattern formation, but a combination of cell-type autonomous and cell-type nonautonomous signaling schemes for controlling cone pattern formation. The cell-type autonomous signals are hypothesized to be inhibitory signals that result in proper local spacing between like cells, whereas the cell-type nonautonomous signals would be permissive or inductive cues that operate at the level of cone photoreceptors, and provide information for subsequent differentiation and patterning of different cone types (see “Mechanisms of Cellular Pattern Formation in the Retina”).

Studies of retinal regeneration suggest that in the vertebrate retina both the cell-inhibitory and cell-permissive signals may be derived from a template of previously differentiated, or currently differentiating, cells. It should be noted that a template of mature neurons is not required for the generation of a specific retinal cell type arising from either normal retinal growth or following retinal injury [104], suggesting the operation of differentiation mechanisms that are independent of pre-existing patterning cues. But once even a single new retinal cell is formed, it may act as a “seed” of cell-permissive and/or cell-inhibitory signals. These signals can then direct the formation of the subsequent cellular pattern.

Such a template mechanism involving immature, or newly born, neurons is consistent with the observation that new retina arising from the CGZ following a chemical lesion does not manifest a proper cone mosaic; the template (i.e., previously-differentiated photoreceptors) had been destroyed by the chemical treatment, precluding proper pattern formation within new generations of cones. The broader aspects of cone pattern, as determined by quadrat analysis, are therefore compromised [65]. However, once new cones are generated some of the “normal” signaling mechanisms can be re-established, permitting the subsequent cone pattern to display local pattern attributes that remain significantly different from random patterns (as objectively determined by NND and DRP analysis).

Pattern irregularities in retina regenerated following a surgical lesion, and in the regenerated retina that arises following a chemical lesion, provide further support to the hypothesis that template-derived signals are necessary for proper cone mosaic formation. After a surgical lesion a regenerative blastema forms along the edge of the wound and appositionally adds new neurons, eventually filling the wound with new retina [74]. In these circumstances, any spatial irregularities along the edge of the regenerative blastema, even very subtle ones, could significantly alter the resultant spatial profile of the inhibitory and permissive signals as compared to the CGZ (Figure 7). Cone photoreceptors of the appropriate type are produced, but the regulation of their spatial pattern is compromised to such an extent that the recapitulation of a regular, square-like mosaic is precluded; local anti-clustering occurs (consistent with the NND and DRP analyses) but the regular pattern is not restored (consistent with quadrat analysis). Because these signaling mechanisms are hypothesized to influence relatively early aspects of cone formation, rather than simply directing the positioning of, for example, opsin-expressing cones that have already been generated, the model might also explain the ubiquitous presence of atypical cone mor-
Cellular pattern formation in retina regenerated after a chemical lesion is also consistent with a template-driven patterning mechanism. Following such a lesion, the entire neural retina is destroyed (but not always; see [65,112]) except for proliferative cells that are scattered throughout central retina and the CGZ [76]. The scattered proliferative cells form scattered regenerative clusters, or foci, that generate new retinal neurons. As proliferative cells extend outward from the foci, leaving new retina in their wake, the fields of new retina may ultimately collide and merge. Based upon the model signaling scheme such a “patchwork” of regenerated retina may be predicted to lack a regular pattern of cones due to the lack of a template-and this is exactly the situation indicated by both visual inspection and quantitative analysis of the regenerated cone mosaic [65]. Interestingly, in this case the regenerated retina not only lacks a regular pattern, but also lacks anti-clustering, and based on quadrant analysis, shows significant clumpiness, indicating that neither broad nor local patterning mechanisms are successfully re-established (Figure 6). If we assume that the density of regenerative foci is similar to the density of rod precursors (or PINCs) prior to the injury, we would predict that adjacent regenerative clusters could merge in fairly short order. In this scenario, signaling mechanisms arising from one cluster could compete, perhaps destructively, with the signals emanating from the adjacent cluster(s). This spatially complex “competition” of molecular signals may result in the aggregated cone mosaic patterns that are frequently observed in regenerated retina following a chemical lesion [65].

**CANDIDATE SIGNALS FOR DEVELOPMENT OF THE VERTEBRATE CONE MOSAIC**

In addition to providing important clues for determining fundamental retinal patterning rules, regenerating teleost retina also now offers a valuable comparative experimental system for testing molecular candidates for these putative signaling schemes. Are there molecular candidates for these putative inhibitory and permissive signals? Candidate agents for mediating inhibitory signals between like-cell types can be based again upon those having this role during *Drosophila* ommatidial development. Known examples are signals that refine the spacing of the putative “founder” photoreceptor (e.g., Scabrous, EGF receptor-mediated signaling, the Notch-Delta system). A potential role for Notch-Delta signaling in teleost cone mosaic development was noted recently by the study of the *mind-bomb (mib)* mutant zebrafish, which has a defect in the Notch signaling pathway [113]. In *mib* embryos, red cone opsin is the only cone opsin expressed [114]. Does this indicate that red cones are the “founder” photoreceptors and, in the mutant, lateral inhibition to restrict founder cell fate has failed? Further studies will be necessary to confirm whether this is the case, but a founder role for red cones has previously been suggested [48] because they are the first cones to differentiate [44-47]. The Notch gene is expressed during retinal neurogenesis following injury [89,100], but involvement in retinal cell patterning has not been directly tested. Indeed, a formal, mechanistic requirement for a cone founder cell in successful cellular pattern formation during vertebrate retinal development or regeneration has yet to be firmly established (see “Development of Retinal Cell Mosaics: Insects and Vertebrates”).

Based upon analogy with the *Drosophila* system, candidate signals for the inductive/permissive, cell-type non-autonomous cues could include homologues of the sevenless-boss interaction and their downstream components, such as the orphan nuclear receptor, seven-up. Repressed function of seven-up causes specification of additional R7 photoreceptor cells at the expense of other photoreceptor types [115]. An addi-

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**Figure 7. Schematic of vectorial growth in the normal and regenerating retina.** Top: In the normal retina growth occurs in an annular fashion, with new cells born at the circumferential germinal zone (red line) and appositionally added to the extant retina, forming a new retinal margin (blue line) that is in spatial register with the old margin. The direction of growth (black arrows), which is locally perpendicular to the tangent of the retinal edge, is spatially aligned with the pre-existing patterns of retinal cells (black dotted lines). Bottom: In this model the edge of a lesion site is quite “jagged” compared to the retinal margin. The vectors of regenerative growth (black arrows), which extend into the lesion site, may be spatially and temporally uncorrelated, and may also be uncorrelated with the pre-existing cellular patterns in extant retina (black dotted lines). These spatiotemporal abnormalities in growth are hypothesized to contribute to the atypical cellular patterns of regenerated retina.
tional set of candidate signaling systems includes those homologous to the *Drosophila* ecdysone/ultraspiracle receptor complex, because ecdysone signaling may be important for position-dependent specification of cone identity. Reduced levels of ecdysone during imaginal disk development result in premature commitment of cells to the R1, R6, and R7 fate and consequent ommatidial pattern disruption [116]. The ecdysone receptor, a member of the steroid/thyroid superfamily of ligand-regulated transcription factors, forms heterodimers with ultraspiracle, the *Drosophila* version of the vertebrate retinoid X receptor [117]. While the function of ecdysone (and related signaling pathways) may not be as a specific inductive agent, ecdysone is likely to regulate the timing of the inductive pathways, to coordinate the onset of expression of the more specific molecular signals [118,119].

In vertebrates, activated retinoid X receptors (RXRs) form homodimers or heterodimers with thyroid hormone receptors (TR), retinoic acid receptors (RAR), or vitamin D receptors [120]. Two activators of these signaling systems, thyroid hormone and retinoic acid, are known to influence vertebrate photoreceptor differentiation and survival in vitro and in vivo [121-127]. Additionally, mice lacking one of the thyroid receptor genes (TR beta 2) have an excess of short wavelength-sensitive (blue) cones relative to medium-wavelength sensitive cones [128], reminiscent of the excess of blue cones observed in regenerated retina and in new retina arising from the CGZ following a chemical lesion. Lastly, the inherited human disorder, enhanced S-cone syndrome, characterized by an excess of blue cones, is linked to a molecular defect in an uncharacterized member of the steroid/thyroid receptor family [129]. Together these studies suggest an important role for retinoids and/or thyroid hormone (or at least their receptors in conjunction with the RXR) in regulating cone determination, and perhaps as a consequence cone pattern formation. Preliminary experiments suggest that an excess of retinoic acid or thyroid hormone during retinal neurogenesis results in photoreceptor pattern abnormalities (unpublished data). Ongoing studies are investigating potential compromises in retinoid/thyroid signaling during retinal regeneration.

**CONCLUSIONS AND SIGNIFICANCE**

Although the precise molecular mechanisms that regulate cellular pattern formation in the vertebrate retina have remained enigmatic, important clues have come from several sources: comparison with photoreceptor patterning in *Drosophila*; descriptive analysis of photoreceptor differentiation in vertebrates; quantitative analysis of retinal cell pattern in mature vertebrate retina; modeling studies of cell patterning; and most recently from the quantitative analysis of retinal cell patterns in regenerated fish retina. These studies are collectively consistent with the following model for vertebrate retinal cell patterning (Figure 8). Position-dependent, cell-extrinsic signaling systems are likely to be involved. At least one signaling system is needed for the formation of inner retinal cell mosaics, and this system is likely to be cell type-autonomous and inhibitory. However, at least two signaling systems are needed for the formation of cone mosaics (such as those in teleost retinas): a cell-type autonomous, inhibitory system, and a cell-type nonautonomous, inductive/permissive system. The signals responsible for retinal cell patterning are likely derived from a template of previously (or currently) differentiating cells. Candidate signaling systems for vertebrate cone patterning, for which some experimental evidence is available, include the Notch-Delta system and the retinoic acid/thyroid hormone systems.

The insights for cellular pattern formation that have been obtained from the study of retinal regeneration are important for two major reasons. Firstly, the vertebrate retina presents itself as a useful model for the study of nervous system assembly, and patterning rules deduced for the retina may have

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**Figure 8.** An inclusive model for the assembly of vertebrate retinal cell patterns. **A**: Inner retinal neurons. A position-dependent, template-derived, inhibitory signaling system (red arrows) prevents like-type (black) cells from occupying nearby space. **B**: Cone mosaic. 1. A position-dependent, template-derived, inhibitory signaling system (red arrows) prevents like-type (black) cells from occupying nearby space. 2. A position-dependent, template-derived, inductive or permissive signaling system (green arrows) allows unlike-type (gray) cells to occupy nearby space. **C**: Regenerated retinal patterns. 1. No template and/or failed inhibitory signaling results in no restrictions on (black) cell position. 2. Poor template and/or failed inductive/permissive signaling results in faulty information for (gray) cell position.
broad applicability elsewhere in the central nervous system. Secondly, an understanding of tissue patterning, and especially of retinal patterning during regeneration in mature organisms, has applications for treatment of human retinal disorders. Because retinal stem cells are now known to reside either near to or within the neural retina of mammals, the replacement of lost cells in humans suffering from retinal degenerative conditions such as macular degeneration or retinitis pigmentosa is now an exciting possibility. Continued investigation of cellular genesis and pattern formation in regenerated fish retina is expected to provide critical information toward understanding how neuroregenerative phenomena might ultimately be harnessed for the restoration of visual function in humans.

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