

Molecular recapitulation: the growth of the vertebrate retina

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ABSTRACT In postembryonic lower vertebrates, the ciliary marginal zone (CMZ) of the retina is a continuously growing zone in the central nervous system. By studying the cellular and molecular biology of the cells in this region, we have discovered that the CMZ can be divided into several zones, from peripheral to central, which reflect different stages of development of retinal stem cells. Based on the behavior of the cells and on the genes expressed in different regions, we propose here that cellular development in the CMZ recapitulates in space what happens in embryonic retinal development in time.

KEY WORDS: *Xenopus*, *Notch*, *Delta*, *atonal*, *pax6*, *six3*, *Rxd*, *Xash*

Stem cells and the recapitulation of development

If anything in developmental biology recapitulates anything else, then perhaps what individual stem cells do in the mature organism recapitulates what their embryonic ancestors did. In many organs systems, including blood, skin, bone, nervous system, muscle, etc., the developmental process gives rise to cells that do not differentiate immediately, or perhaps ever. These cells may continue to divide throughout much of the lifetime of an animal, giving rise, as their embryonic ancestors did, to mature organ derivatives and further self-renewing stem cells. Within a single asymmetric cell division, a stem cell in an adult can turn into another stem cell like itself that has indefinite rounds of division and no differentiation in its offspring, and a different type of cell that has a limited number of cell divisions in its. Embryos generally go from simple to complex, immature to mature, undifferentiated to differentiated. Stem cells of mature organisms also follow time's arrow, and when they spin off progeny that are more differentiated, it is rare indeed that such progeny will dedifferentiate and become stem cells again.

While there are general parallels between the development of zygotes and that of stem cells, there is also a molecular recapitulation of development in the division and determination of stem cells and their progeny. This idea, though probably not new, presented itself with forcefulness when we began to study the initial molecular and cellular development of the frog retina, on the one hand, and genes that are expressed in the stem cells at the ciliary margin of the retina, on the other.

Developmental sequence is spatially arrayed in the ciliary marginal zone

The eyes of frogs and fish grow throughout life, remaining in proportion to the size of animal (Straznicky and Gaze, 1971; Johns,

1977). Over the years, a goldfish or frog may increase in mass a million fold or more, and the cell numbers in the eye increase proportionally (Fig. 1A-C). The new cells are not interspersed but added at specific germinal zones. In the spherical lens, this is at the equatorial circumference. In the two dimensional disc of retina, new cells are added in the ciliary marginal zone (CMZ), a peripheral ring of undifferentiated cells located at the extreme perimeter (Fig. 1D-E). As the animal ages, new rings of cells are added, and the retina grows by accretion at the outer edges. A single pulse of a birthdate marker, such as tritiated thymidine, specifically labels the CMZ (Johns, 1977). Pulses of BrdU or tritiated thymidine, separated by intervals of a week or more, label rings of cells in the retina, suggesting that the postmitotic descendants of the CMZ do not migrate, but remain in place while the next cells are added more peripherally. The spacing between the rings gives an indication of the rate of growth, like the thickness of tree rings. In a rapidly growing adult goldfish, thousands of new cells are added every day. About a quarter of these are ganglion cells that must send axons through the optic nerve into the CNS and then make connections with the tectum while preserving a topographic order in the retinotectal map, a process that amazingly requires the continual breaking and reconnecting of all the synapses in this system (Straznicky and Gaze, 1971; Gaze *et al.*, 1979; Fraser, 1983). In an adult fish or frog, the most central area of the retina is made of the cells that are the oldest, born during embryogenesis, and as one moves laterally, one samples cells born later and later in the animal's life.

The most peripheral or deepest part of the CMZ is where the pigment epithelium folds over into the retina. Stem cells in this region give rise to both neural retina and pigment epithelium, just as cells of the early eye field give rise to both structures (Wetts *et al.*, 1989). The most peripheral cells in the CMZ, when injected with a non dilutable lineage tracer, often generate clones of cells

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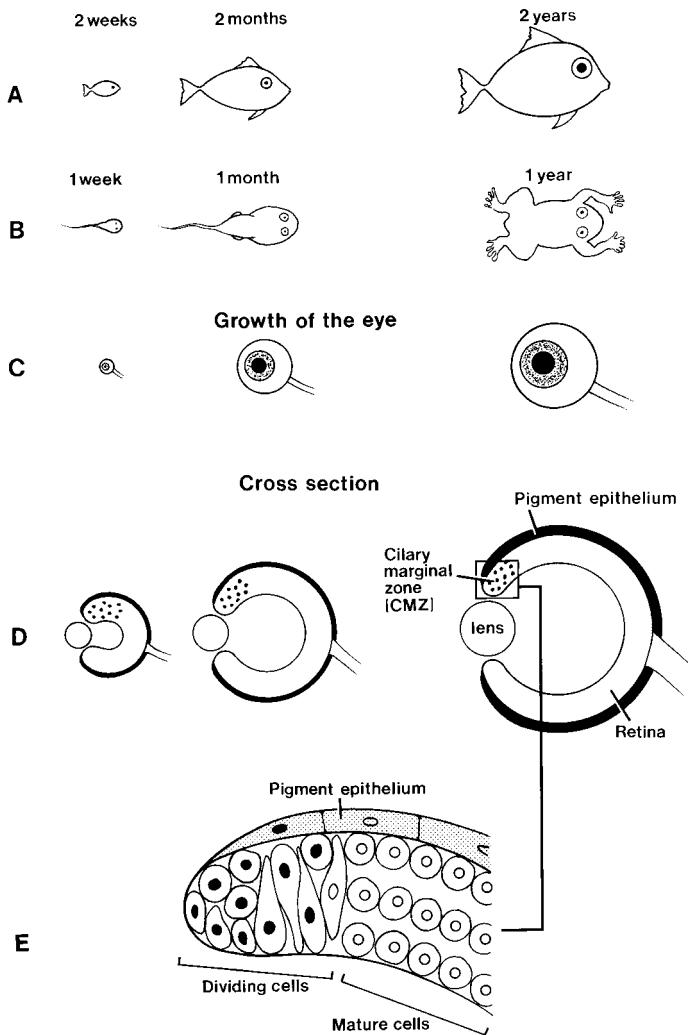


Fig. 1. Postembryonic growth of the eye through cell division at the ciliary marginal zone in lower vertebrates. The top two panels show the postembryonic growth of a fish (A) and a frog (B) over a period of a year or more. (C) The eye grows concomitantly with the animal, and (D), a cross section through the retina shows labeled nuclei of dividing cells which are added at the ciliary marginal zone (CMZ) at the periphery of the retina. (E) Undifferentiated dividing cells of the CMZ, as they abut the unlabeled (open nuclei) postmitotic laminated retina.

that radiated from the periphery toward the center in both the retina and pigment epithelium (Fig. 2A), suggesting a trail of postmitotic progeny from a single self renewing stem cell (Wetts *et al.*, 1989). When, however, dividing CMZ cells slightly central to these deepest cells were labeled, the result was usually a small clone of cells in the retina (Fig. 2B), that does not extend to the periphery (Wetts, *et al.*, 1989). One can thus imagine a gradient of development in the CMZ from peripheral to central. The most peripheral cells are deep stem cells, able to give rise to retina, pigment epithelium, and other stem cells. The next most central cells in the retina, the progeny of these deep stem cells, are still mitotic, but are retinoblasts. They do not give rise to further stem cells, but do generate clusters of cells containing a variety of neural and non neural retinal cell types. These retinoblasts of the

CMZ bear a striking resemblance to embryonic cells of the retina primordium at the optic vesicle stage that give rise to small clones of cells of many retinal cell types (Holt *et al.*, 1988; Wetts and Fraser, 1988). The later in developmental time one labels these retinoblasts, the smaller the clone size (Holt *et al.*, 1988). Using this procedure one can estimate the cell cycle time, which agrees with estimates based on the increasing numbers of cells in the primordium during these stages, and one can predict the number of cells in the primordium at earlier stages, as has been done in *Drosophila* using mitotic recombination to generate marked clones at successively later stages (García-Bellido and Merriam, 1971). The most central cells of the CMZ (Fig. 2C), although they appear undifferentiated, do not take up thymidine analogs, and so presumably have left the cell cycle, and are likely to be in the process of determining which cell type to adopt (Dorsky *et al.*, 1995). This temporal gradient of cells in the CMZ thus continues the gradient in the retina, the most mature or furthest developed progenitors being the most central and the least mature, deepest stem cells being the most peripheral.

Like the eye of a fly, the eye of a frog develops largely according to an American rather than a European style, lineage being less important than neighborhood. If an embryonic retinoblast is labeled with a vital dye, the composition of the clone cannot be predicted, (Holt, *et al.*, 1988; Wetts and Fraser, 1988). Thus when clones consist of two cells, knowledge of one sibling's cell type is not helpful in predicting the identity of the other. From this we derive the hypothesis that extracellular interactions, perhaps with neighboring cells—as in the fly eye—may be critically important in determining cell type. Direct evidence for this hypothesis comes from experiments in cell culture where embryonic retinoblasts have been dissociated and reaggregated with other cells of different age and type (Adler and Hatlee, 1989; Reh and Kljavin, 1989; Watanabe and Raff, 1990; Altshuler *et al.*, 1991; Harris and Messersmith, 1992). Based on these experiments, short range interactions are shown to play a key role in cell type determination. In addition, feedback mechanisms operate to assure that the correct proportions of cells in each class are made. In the embryonic retina, for example, the first cells to be generated, the ganglion cells, inhibit other cells from choosing the same fate (Waid and Mcloon, 1998). Thus grafting ganglion cells from an older retina onto a younger neuroepithelium, inhibits the normal generation of ganglion cells in that epithelium. Here the CMZ is again strikingly similar to the embryonic retina. The overproduction of dopaminergic amacrine cells from the CMZ can be induced following selective killing of these cells in the mature central retina using neurotransmitter analog toxins (Negishi *et al.*, 1982; Reh and Tully, 1986). The extra amacrine cells near the edge of the retina then feedback again to the CMZ, which in the following days makes less than the normal number. Eventually these oscillations level out and the normal rate of amacrine cell generation is restored.

The spatial gradient of stages in the CMZ, from peripheral to central, i.e., from deep stem cells at the extreme periphery, to retinoblasts slightly more centrally, to postmitotic but undifferentiated progenitors at the central edge of the CMZ, is perhaps the most interesting feature of this system in that it maps development in space rather than time (Fig. 2D). This spatial gradient of cellular properties led us to predict that the expression of the genes in the CMZ would define a similar gradient, recapitulating in space the temporal gradient of gene expression in retinal development.

Molecular recapitulation: four zones of development

Specification zone

What are the earliest genes expressed in the retinal primordia? At the late gastrula/early neural plate stages of development, a few key controllers of eye development are first expressed in the single anterior eye field at the front of the neural plate: *Six3*, *Pax6*, *Rx1* (Casarosa *et al.*, 1997; Hirsch and Harris, 1997b; Mathers *et al.*, 1997; and Zuber *et al.* in preparation). All of these are homologous to *Drosophila* genes that are expressed in the eye primordia or disc and are critically involved in the development of the compound eye. The molecular parallels between the genes involved in the development of vertebrate and invertebrate eyes is an amazing story in itself because it challenges the long held view, supported by embryological, physiological, and anatomical data, of multiple distinct origins of eyes in the animal kingdom (Land and Fernald, 1992; Halder *et al.*, 1995; Harris, 1997). Not only is the expression of all three genes largely confined to the eye primordia in the embryo, but also even in the more mature animal, the expression of these genes is still restricted to the eye. In the retina, all of these genes are expressed throughout the CMZ, even in the most peripheral regions (Fig. 3A) and also in distinct layers of the central retina, as though these gene might be used in two phases, first in the development of the eye character in the anterior neuroepithelium, and second in the development of specific cell types in the retina (Perron *et al.*, 1998). The expression of *Pax6* is particularly suggestive in this regard as it is clearly upregulated with respect to the CMZ in the ganglion and amacrine cell layers (Hirsch and Harris, 1997b).

Proneural and neurogenic zone

When the nervous system is induced during normal development, a set of proneural genes are turned on in the presumptive nervous system. Among these are the vertebrate homologs of the *Drosophila achaete-scute* homologs, the *ash* genes, which were first discovered to have a proneural role in flies (Ghysen and Dambly-Chaudiere, 1988). *Xash1* and *Xash3* are both expressed in the embryonic eye and in the CMZ of the mature eye (Ferreiro *et al.*, 1993; Zimmerman *et al.*, 1993). During embryogenesis, these genes are expressed in the eye field slightly after *Pax6*, *Six3* and *Rx1*. In the CMZ, we discovered that, unlike the eyefield specific genes, they are not expressed in the most peripheral or deepest part of the CMZ (Fig. 3B), where the stem cells that can give rise to the neural retina and the non-neural pigment epithelium are localized (Perron and Harris, 1997). The other, central, border of *Xash1* expression extends to the approximate central edge of the CMZ, past the last proliferating retinoblasts, but does not extend into the fully differentiated layered retina. *Xash3* expression tapers off more peripherally. One function of the *Xash* gene products in the CMZ presumably is to promote neural character to the retinoblasts, which by virtue of expressing eye-specific genes already have eye-specific character.

Another function of the *Xash* genes may involve the regulation of the neurogenic genes. The neurogenic genes are members of a cell-cell signaling system and include the signaling molecule *Delta*, its receptor *Notch*, and at least some of the nuclear effectors of this signaling pathway, the *Enhancer-of-Split* gene products (Muskavitch, 1994). A function of the neurogenic genes in flies and vertebrates seems to be to inhibit them from differentiating in response to the other local signals that drive cells toward particular

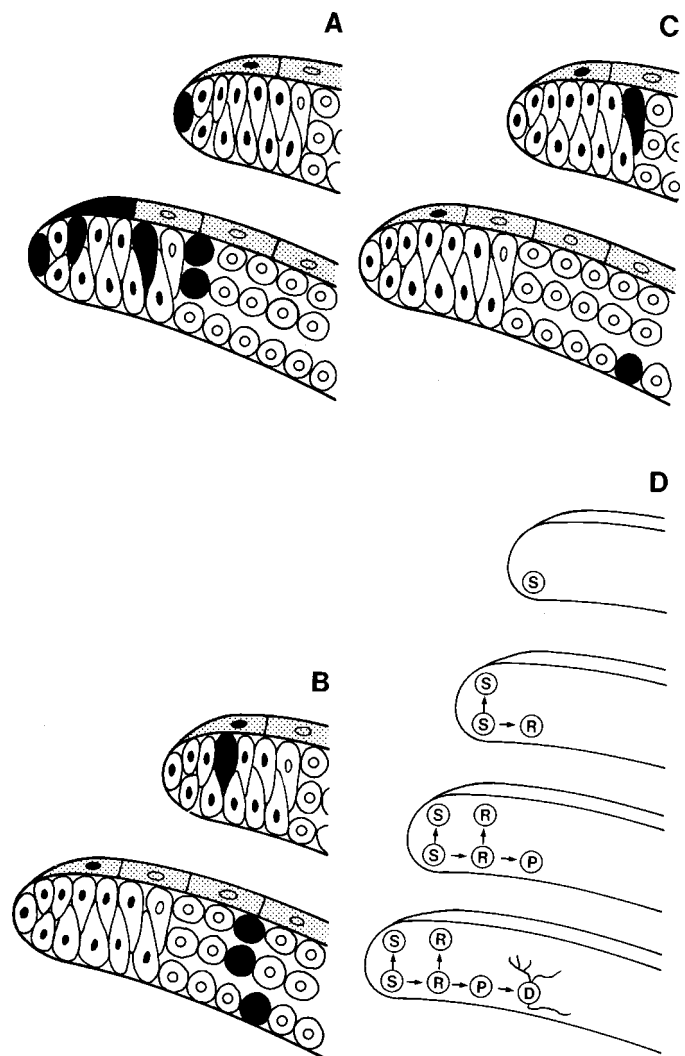


Fig. 2. Progressive development in the CMZ as demonstrated by cellular division and differentiation of marked cells. In (A) a very peripheral cell is labeled (top) and the progeny it gives rise to are shown (below) after some growth at the CMZ. In this case the cell is a stem cell that regenerates itself as well as giving rise to a continuous stream of retinal and pigment epithelial cells. In (B) a cell in the middle of the CMZ is labeled (top) and after, it divides only a couple of times and give rise to a cluster of postmitotic daughters (below). An undifferentiated cell at the central edge of the CMZ is labeled in (C) (top). It does not divide further but simply differentiates into a neuron in the laminated retina. In (D), a model of progression from a single stem cell at the periphery is shown. The single stem cell (S, top) gives rise to another stem cell and a retinoblast (R, second from top, although it may also give rise presumably to a pigment epithelial progenitor). This retinoblast gives rise to another retinoblast and a postmitotic but undifferentiated progenitor (P, second from bottom). The bottom panel shows the progenitor giving rise finally to a differentiated neuron (D).

fates (Coffman *et al.*, 1993). The entire set of neurogenic genes is expressed in concert with the *Xash* genes in the CMZ, starting at the same peripheral edge of *Xash* expression and turning off at the same central edge. Thus, one can imagine the cells in this zone of the CMZ as being pushed to become neural from the *Xash* genes, while at the same time being inhibited from differentiation by cell-

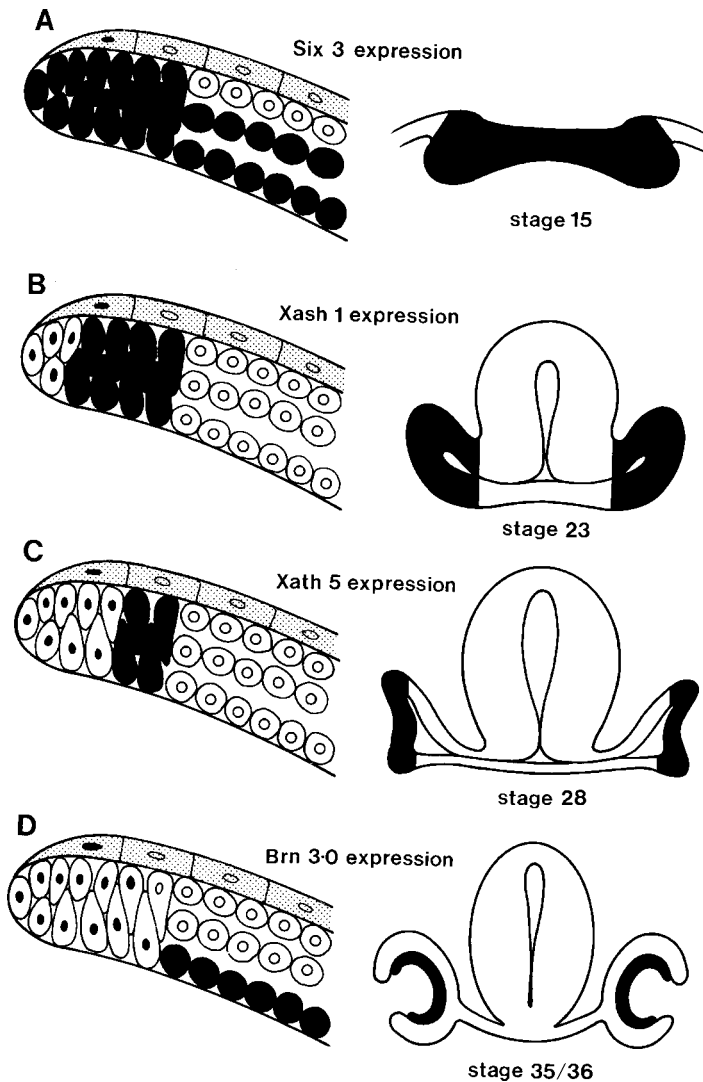


Fig. 3. Progressive gene expression in the postembryonic CMZ recapitulates the embryonic order of gene expression in the retina. (A) *Six3* expression in the postembryonic CMZ starting at the peripheral edge (left) and in the eye fields of the stage 15 neural plate (right). **(B)** *Xash1* expression starting slightly less peripherally and extending centrally to the edge of the CMZ (left), and in the optic vesicle of a stage 23 embryo. **(C)** Expression of *Xath5* in the most central zone of the CMZ and in the retinal primordium of the stage 28 optic cup. **(D)** *Brn-3.0* is only expressed in postmitotic differentiated cells as they leave the CMZ. It is also expressed in these cells when they differentiate in the embryonic retina at stage 35/36.

cell signaling through *Notch* and *Delta*. At the central edge of the CMZ, the neurogenic pathway is downregulated, and so one idea is that only the cells at this central edge, that are now immune from lateral inhibition, are allowed to respond to the local cell determination signals and begin to differentiate. By thus controlling the number of cells allowed to differentiate at any particular time and place, the nervous system gains a finer control on the generation of cell diversity.

Cellular determination zone

There are no generic neurons in the CNS. Each cell has a particular brand name or identity. In the retina, cells are, for

example, one of a variety of ganglion cell types. The *Xash* genes may help promote a neural identity, but there is little evidence in flies or vertebrates that the *achaete scute* class of transcription factors promote further specification. Rather this is the job of genes further downstream, such as the *atonal* homologs. In *Drosophila*, *atonal* is involved on specification of the photoreceptor cells in the eye (Jarman *et al.*, 1995). In vertebrates, there are several *atonal* homologs. In *Xenopus*, for example, we know of *NeuroD*, and *ATH-3*, and *Xath5* that are expressed in the retina and clearly turn on even later in retinal embryogenesis than do the *Xash* genes (Lee *et al.*, 1995; Kanekar *et al.*, 1997; Takebayashi *et al.*, 1997). In the CMZ, *ATH-3*, *Xath5* and *NeuroD* are all expressed less peripherally than the *Xash* genes (Fig. 3C), indicating that they come on later in development (Perron *et al.*, 1998). Functional analysis, involving misexpression of dominant *Xash*, dominant neurogenic genes, or some *atonal* homologs in *Xenopus* embryos, shows that genes such as *neuroD* and *Xath5* act downstream of the *Xash* genes. *NeuroD* is upregulated by *Xash3* and, unlike *Xash3*, *NeuroD* at least is functionally insensitive to lateral inhibition through the neurogenic pathway (Chitnis and Kintner, 1996; Kanekar, 1997). This is very like the *asense* gene in the sensory organ precursors in *Drosophila* (Brand *et al.*, 1993). In addition to these basic helix loop helix genes, X-MyT1 is a zinc-finger transcription factor that in co-operation with bHLH proteins also promotes neurogenesis (Bellefroid *et al.*, 1996). Its expression is turned on in the CMZ at the same level with the *atonal* homologs. While still expressed in dividing cells of the CMZ, three of these four continue to be expressed in specific layers of the mature central retina, suggesting that they have roles in maintaining as well as initiating specific determined states (Perron *et al.*, 1998). In accordance with a role in promoting the development of a specific cell type, transfection of the developing retina with *Xath5* causes misexpressing cells to tend to assume a ganglion cell fate (Kanekar *et al.*, 1997), although the promotion of this specific fate might also be because *Xath5* promotes early neurogenesis in a context where the first cells to differentiate tend to become ganglion cells by default. Thus, the expression of the *atonal* homologs defines a third region of the CMZ, beginning not only after (more central than) the expression of the eye specific genes, but also after (more central than) the proneural *Xash* and neurogenic genes.

In this third region, another gene, *Otx2*, is also expressed. A homolog of the *Drosophila orthodenticle* gene involved in head specification as well as the specification of particular neuroblast in the CNS, *Otx2* is expressed not only in the retina, but in other regions of the frog CNS (Finkelstein *et al.*, 1990). Mice heterozygous for *Otx2* show severe eye defects (Matsuo *et al.*, 1995). Like some of *atonal* homologs, *Otx2* remains on in the specific layers of the mature central retina (Perron *et al.*, 1998). The genes expressed in this third zone, possibly in combination with other genes that remain on, may help establish tendencies in the proliferating retinoblasts to assume certain fates when these cells are released from lateral inhibition through the neurogenic pathway.

Differentiation zone

The most central or fourth region of the ciliary margin is distinguished not by the expression of any of these genes, but by the fact that cells are all non-dividing. This most central region of the CMZ thus represents the most advanced of the undifferentiated cells that have turned on whole batteries of genes, have

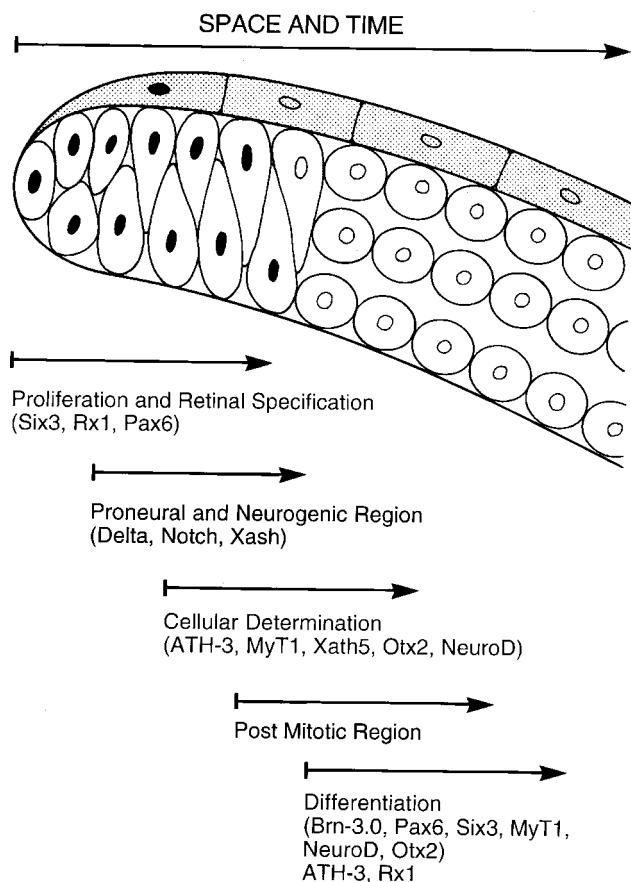


Fig. 4. Model of molecular development in the CMZ. The array of cells from left to right in this model of the CMZ can also be considered as a temporal sequence of gene expression. The genes involved in retinal specification are expressed at the peripheral edge of the CMZ and represent the earliest zone of development. In the next zone, the proneural and neurogenic genes are expressed pushing retinal cells toward a neural fate but keep them undetermined with respect to cell type. In the next zone, the cellular determination genes such as the *atonal* homologs are expressed. The next zone represents cells that have stopped proliferating although they are still undifferentiated and express most of the above genes. The final zone represents the maturation of the postmitotic cells into retinal neurons where they express genes that are constitutively on in fully differentiated neurons.

pulled out of the cell cycle, are juxtaposed to the mature neurons of the central retina and so are in a position to receive signals from their membranes, but yet have not overtly differentiated.

From the fourth region of the CMZ, the next older cells in the retina are mature differentiated neurons. At this transition, the CMZ cells abruptly turn off *Xash*, *Notch*, *Delta*, *ESR1*, and *ESR3* genes, i.e., all the *achaete-scute* homologs and neurogenic genes in all cells. In addition, the eye determination genes, the *atonal* homologs, *MyT1*, and *Otx2*, are turned off centrally in those layers or sublayers where they are not expressed (Perron *et al.*, 1998). The final demarcation of the end of the CMZ is that new genes are turned on as the neurogenic genes are turned off. A particularly good example of this is the *Brn-3.0* gene in *Xenopus*. The *Brn-3.0* gene, originally studied in mice, has been shown to be important for the

ultimate differentiation or survival of ganglion cells (Erkman *et al.*, 1996; Gan *et al.*, 1996). In the embryo, *Brn-3.0* is not expressed in the retina until the late stage of optic cup formation, after the first ganglion cells have pulled out of the mitotic cycle and after the expression of genes like *Xath5* (Hirsch and Harris, 1997a). This positions *Brn-3.0* as the last developmentally expressed transcription factor discussed here. In accordance with this, *Xenopus Brn-3.0* is expressed in ganglion cells throughout the central retina right up to the border of the CMZ, but it is expressed in no cells of the CMZ, even the postmitotic ones, that express the neurogenic genes (Fig. 3D).

Conclusions

The retinal CMZ of lower vertebrates is a powerful system to study the genetic pathway of neurogenesis in vertebrates. The spatial ordering of genes, from peripheral to central, reflects a developmental sequence, and suggests a developmental cascade. Such developmental ordering of expression is similar to that seen in the morphogenetic furrow region of the *Drosophila* eye which bears a striking similarity to the CMZ. The proneural and neurogenic genes are expressed in stripes that parallel and overlap the morphogenetic furrow in the eye disc. The *atonal* gene is expressed in a stripe at the anterior edge of furrow and then becomes restricted to R8 photoreceptors (Jarman *et al.*, 1994). This can be closely compared to the *Xenopus neuroD*, which is first expressed throughout the third and fourth zones of CMZ and its expression becomes confined to the photoreceptor layer in the retina. Because of these striking parallels in cellular and molecular biology, we propose that the CMZ is a vertebrate counterpart to the morphogenetic furrow of *Drosophila*. Unlike the case in *Drosophila*, where the morphogenetic furrows sweeps across the eye disc once in the building an eye, the CMZ is a perpetually developing "furrow" in which the relative spatial order of gene expression, from peripheral to central, recapitulates the order of gene expression in the rapidly developing embryonic retinal primordium. The succession of gene expression, so clearly delineated in space (Fig. 4), allows us to see the steps of molecular development arranged in a single linear dimension, and thus provides clear models of which genes are upstream of others. Some of these predictions have been born out by independent functional analysis. Surely, there are many other genes that turn on and off in the CMZ, and perhaps analysis of these will lead to further subdivision of the CMZ, illustrating in fine detail the developmental genetics of the vertebrate retina, as other such proliferative zones may in turn shed light on the particular development of other areas of the CNS.

The spatially arrayed system of stem cells, retinoblasts, and precursors in the CMZ has allowed us to formulate parallels with the morphogenetic furrow of *Drosophila* on the one hand, and more importantly with normal development of the retina on the other. The challenge for the future will be to interfere with gene expression in the CMZ using molecular strategies, in order to test the potential of these cells and the precise function of the genes they express along this developmental dimension.

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