The Effects of Natural Cell Loss on the Regularity of the Retinal Cholinergic Arrays

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The retina provides a paradigmatic example of the modularity of neuronal circuitry. Different cells are stacked in layers, and neurons of the same type are commonly regularly spaced within their layer. Although the orderly arrays formed by homotypic neurons provide the basis for parallel processing, the mechanisms responsible for regular cell spacing are just beginning to be elucidated. All the developing retinal arrays for which early markers have been identified are regular before being complete. This indicates that the positional constraints controlling mosaic formation are active at times when cell genesis, migration, and death also occur in the retina. To begin investigating how these different processes are coordinated, we have focused here on the effects of cell death on the spatial organization of the two rat cholinergic mosaics, the only arrays for which the development of spatial ordering has been described quantitatively to date. We have chosen an age interval when new cell genesis is over and death predominantly or nearly exclusively controls cell number in one of these array. We found that the regularity of this array is not improved by the loss of cells occurring in this age period. Rather, death appears to be largely independent of cell position.

Key words: neuronal death; rat; retina; Delaunay segments; autocorrelation analysis; acetylcholine

In the vertebrate retina, a limited number of neuronal types are organized in sequential layers underlying serial processing. Within each layer neurons of the same type are commonly regularly arranged, to provide a homogeneous and complete tiling of the retinal surface (Dowling, 1987; Wässle and Boycott, 1991; Cook and Chalupa, 2000). These neuronal arrays are commonly known as mosaics, for the extreme regularity of cell positioning that some of them display (Wässle and Riemann, 1978).

Recent studies have shown that many developing retinal arrays are regular before all their elements have migrated into their layer, and in some instances before the last array cells have been generated (Larison and Bremiller, 1990; Wikler and Rakic, 1991; Raymond et al., 1995; Cepko, 1996; Bumsted et al., 1997; Galli-Resta et al., 1997; Wikler et al., 1997; Galli-Resta, 1998). These findings place the control of cell positioning during retinal development under a new focus. Being active at times of ongoing cell genesis, death, and migration (Braekevelt and Hollenberg, 1970; Cepko, 1993; Gilbert, 1994), positional constraints may feedback on all these processes, and vice versa. As a contribution to dissecting the complex relationship between the events that sculpt the mature retinal architecture, we have focused in this study on the effect of natural cell loss in one of the retinal cholinergic mosaics.

The cholinergic amacrine cells form two well characterized arrays early in development and contribute to the spreading of the spontaneous electrical activity that shapes the visual circuitry (Feller et al., 1996; Penn et al., 1998). The two cholinergic arrays are located in two separate layers of the retina (Masland and Tauchi, 1986). Both arrays are regular before containing all their elements. Furthermore, the geometry of these arrays is actively preserved as new elements add to the arrays and the retina simultaneously grows (Galli-Resta et al., 1997). Local cell displacement is observed at these times (Reese et al., 1999), suggesting that cells move laterally to maintain their regular spacing as new elements arrive (Galli-Resta et al., 1997; Reese and Tan, 1998). However, death of wrongly positioned elements could also contribute to array regularity, as it appears to be the case in the segregation and refinement of the cat α ganglion cell mosaics (Jeyarasasingam et al., 1998). Death is likely to affect retinal cells well before their number begins to decrease (Galli-Resta and Ensini, 1996). However, as a first approach here we analyze the late phase of mosaic development, when cell addition is over or nearly so, and the number of array cells decreases as a consequence of naturally occurring cell death.

MATERIALS AND METHODS

Experiments were performed on Long–Evans hooded rats in compliance to the national regulation on animal experimentation. Eye collection, dissection, fixation, mounting, and immunostaining were performed as described (Galli-Resta and Ensini, 1996; Galli-Resta et al., 1997). For each age considered we analyzed six retinas: in three of these the cholinergic cells were identified by a polyclonal anti-ChAT antibody

Received Sept. 13, 1999; revised Nov. 3, 1999; accepted Dec. 1, 1999.

This study was supported by the Consiglio Nazionale delle Ricerche and the European Commission, DGXII. We thank D. Petracchi for useful discussions, G. Resta for computer programming. and G.C. Cappagli for technical assistance.

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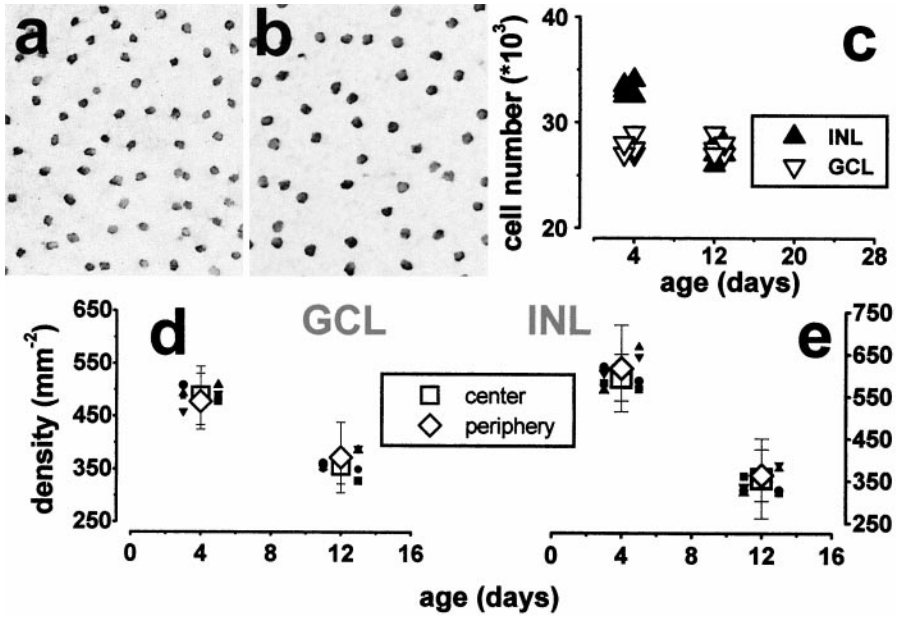

Figure 1. Variation in the number and density of cells in the cholinergic arrays between P4 and P12. *a, b,* Examples of the INL array of cholinergic amacrine cells on P4 (*a*) and P12 (*b*). Both samples are from the temporal retina, taken at mid-eccentricity. Calibration bar, 60 μm. *c,* Whereas the number of cells in the GCL cholinergic arrays does not change significantly between P4 and P12, the INL cholinergic arrays loses ~20% of its cells. This is observed by labeling the cholinergic cells with ChAT (*symbols* with abscissa 4 and 12 d) or with Islet-1 (*symbols* slightly off-centered with respect to the correct age and abscissa). All data have a SD of ≤15%. *d,* The density of cholinergic cells within the GCL decreases between P4 and P12, because the total number of cells in the array is constant, whereas the area of the retina increases. At either age analyzed, the density of cells in the GCL cholinergic array does not vary between the central (*open squares*) and the peripheral half (*open diamonds*) of the retina. Nor does cell density vary significantly with retinal orientation, as shown by the *small filled symbols* on both sides of the open symbols. Symbol code as follows: *triangles,* dorsal; *circles,* ventral; *inverted triangles,* nasal; and *squares,* temporal. The *small symbols* to the *right* of the open ones refer to measures performed in the central portion of the retina; symbols to the *left* refer to the peripheral retina The independence of cell density from retinal location, observed both at P4 and at P12, suggests an isotropic (the same in all directions) growth of the retina. *e,* The density of cholinergic cells in the INL array is similarly plotted, symbols as in *d*. At either age cell density does not vary across the retina. This suggests that the cell loss observed between P4 and P12 is homogeneously distributed across the retina.

(Chemicon, Temecula, CA), whereas in the remaining three retinas, obtained from animals that had undergone optic nerve section at birth (Perry et al., 1983), we used an anti-Islet-1 antibody (a gift of T. Jessel). Once ganglion cells are removed by optic nerve section (Perry et al., 1983), Islet-1 allows to identify univocally the cholinergic cells (Galli-Resta et al., 1997). Samples of both cholinergic arrays were taken at regularly spaced locations across the entire extent of whole-mounted retinas, using a Leica (Nussloch, Germany) TCNS confocal microscope. Sampling fields were $600 \times 600\ \mu m^2$. Between 1/10 and 1/3 of each retina were sampled. Retinas were drawn before and after reaction, and after confocal analysis to control for tissue shrinkage or compression. Sampled fields and retinal drawings were fed to an Image analyzer (Imaging, Ontario, Canada) to obtain cell density, cell positioning, and retinal area. The total number of cells was determined as the average density times the retinal area. Nearest neighboring distances, Delaunay tessellation, and autocorrelation analysis of the sampled arrays were obtained as previously described (Galli-Resta et al., 1999). Delaunay segments link each cell to cells with adjacent domains; the cell (Voronoi) domain includes all the points in the plane closer to the cell than to any other element of the array (Grumnbaum and Shephard, 1989). Plots and frequency histograms were produced by means of Origin 5.0 (Microcal). Simulations based on random cell elimination were obtained by means of a custom-made program. The program was fed with the cell coordinates of each real sampled field and provided an output file in which 20% of the input coordinates had been removed by means of a random generator. The program was tested for randomness on 10×10 crystalline square arrays. The probability of each array element to be removed was found to be constant (with a tolerance of 5%) when 10,000 outputs of the program were analyzed. Ten simulations were performed for each real field. Isotropic stretching was simulated by multiplying all cell coordinates by the same factor.

RESULTS

We found that the cholinergic arrays (Fig. 1*a*, P4, *b*, P12) reach their maximum number of cells on postnatal day 4 (P4). The array located in the ganglion cell layer (GCL) maintains its cell complement constant thereafter, whereas the number of cells in the inner nuclear layer (INL) array decreases in the next few days (Fig. 1*c*). The decrease in the number of cells in the INL array is observed by labeling the cells for either choline acetyltransferase (ChAT) or Islet-1 (Fig. 1*c*), an alternative marker for the cholinergic cells (Galli-Resta et al., 1997). Between P4 and P12 the retina increases in area by a factor of 1.4 ± 0.3. At both ages the density of cells in either array is largely independent of the retinal location (Fig. 1*d,e*). This indicates that the retina grows in an isotropic manner. Otherwise the density of cells in the GCL array would not remain independent of the retinal location. For similar reasons, the cell loss observed between P4 and P12 in the INL array has to be homogeneously distributed across the retina.

Cell loss does not improve array regularity

To understand the effects of cell loss on array regularity, we investigated the spatial organization of the INL cholinergic array, which loses 20% of its cells between P4 and P12 (Fig. 1*c*). A classical measure of array regularity is the regularity index (or conformity ratio), defined as the ratio between the mean and the SD of the distribution of the distance between nearest neighbors (NN) in the array (Wässle and Riemann, 1978; Cook, 1996). The average regularity index is 4.3 ± 0.3 on P4 and 3.9 ± 0.6 on P12. This difference is hardly significant at the statistical level and indicates that death has not improved mosaic regularity at this stage. In addition, cell loss between P4 and P12 has caused a twofold increase in the variability of the regularity index across the retina (it rises from 7 to 15%). The distribution of nearest neighbor distances obtained on P12 (Fig. 2*a, thick line*) is not more narrowly peaked than that obtained on P4 (*thin solid line*), nor is it narrower than the distribution obtained if the P4 cell array is isotropically stretched by the factor the retina grows between P4 and P12 (*dotted line*).

We have extended this analysis to the Delaunay tessellation associated to the array. The Delaunay triangulation is obtained by defining for each cell in the array a domain containing all the points in the plane that are closer to that cell than to any other cells in the array. The Delaunay segments link cells with adjacent domains. Therefore, the Delaunay segments measure the spacing between each cell and its closest surrounding neighbors in the

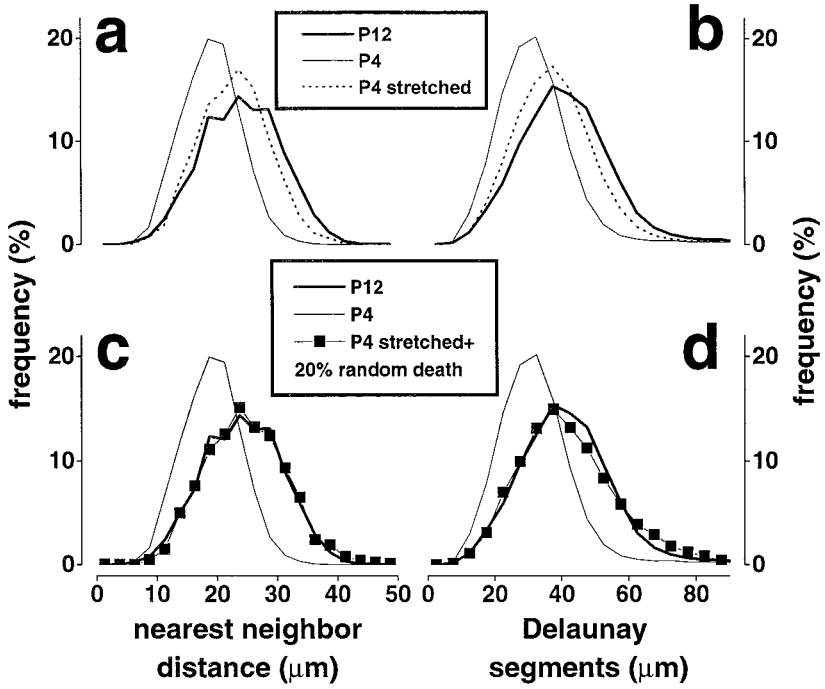

Figure 2. Cell loss does not improve the regularity of the INL cholinergic array. *a,* Nearest neighbor distance distribution for the INL cholinergic array on P4 (*thin line*) and P12 (*thick line*). The distribution obtained on P12 is not narrower than the nearest neighbor distribution obtained by stretching isotropically the P4 fields by the same factor the retina grows (*dotted line*). This suggests that cell loss does not improve mosaic regularity. *b,* Similar conclusions derive from the analysis of the Delaunay segment distribution associated to the mosaic. Symbols as in *a*. *c,* The distribution of nearest neighboring distance on P12 (*thick line*) is very similar to the corresponding distribution (*squares*) obtained with computer simulations where 20% of the cells are randomly eliminated from each real field and the cell coordinates are then isotropically expanded to simulate a 1.4-fold growth in area, corresponding to the retina expansion between P4 and P12. *d,* A good correspondence is also observed between the real P12 (*thick line*) and the simulated (*squares*) distribution of Delaunay segments. The simulation does not provide a perfect fit of the data, but this should not be surprising, considering the use of averaged values for the retinal expansion factor and the amount of cell loss.

array, characterizing the two-dimensional organization of the array as the NN distance distribution cannot do. The distribution of Delaunay segments shows no sharpening between P4 and P12 (Fig. 2*b*). The same considerations apply when the central and peripheral fields are analyzed separately (Fig. 3*a–d*). Thus, cell loss between P4 and P12 does not sharpen the distribution of nearest neighbor or Delaunay segments, indicating that the observed decrease in the number of cells does not lead to an improvement of array regularity.

Cell loss is largely independent of cell position

We have used computer simulations to investigate whether cell loss has any relationship to cell positioning in the array. To this purpose we randomly eliminated 20% of the cells in each sampled field and stretched it by means of an isotropic expansion by a factor of 1.4, corresponding to the increase in retinal area between P4 and P12. The assumption of isotropic growth appears justified by the persistence between P4 and P12 of the independence of cell density from retinal location in the GCL array

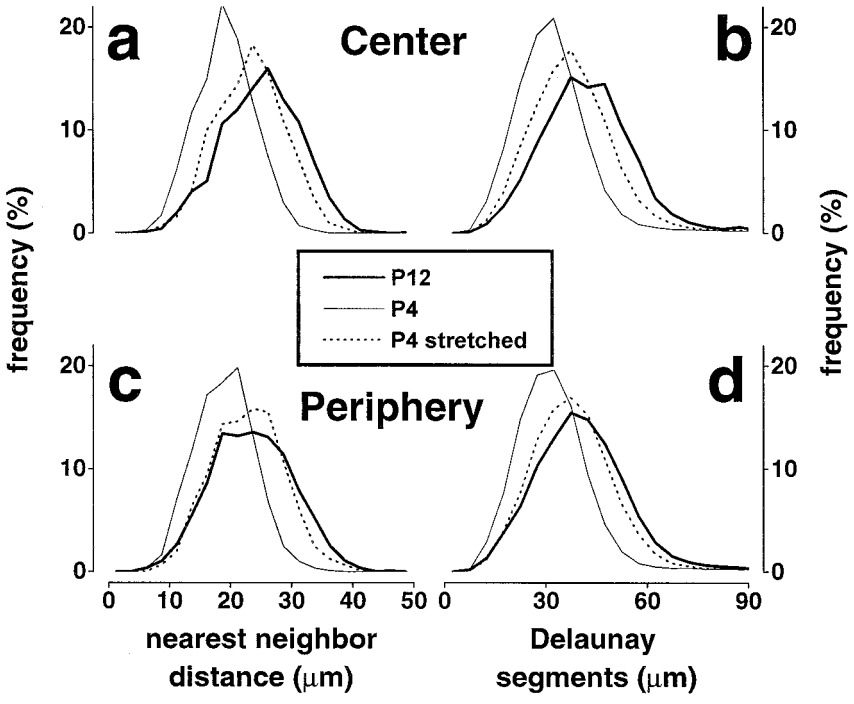

Figure 3. Cell loss does not improve the regularity of the INL cholinergic array in the central or in the peripheral retina. The separate analysis of the nearest neighbor distribution (*a, c*) and the Delaunay segment distribution (*b, d*), associated to central (*top*) and peripheral (*bottom*) fields, confirms that the P12 distributions (*thick lines*) are not sharper than those obtained by simply stretching the P4 fields to simulate retinal growth between P4 and P12. This confirms that cell loss does not improve the regularity of the INL array.

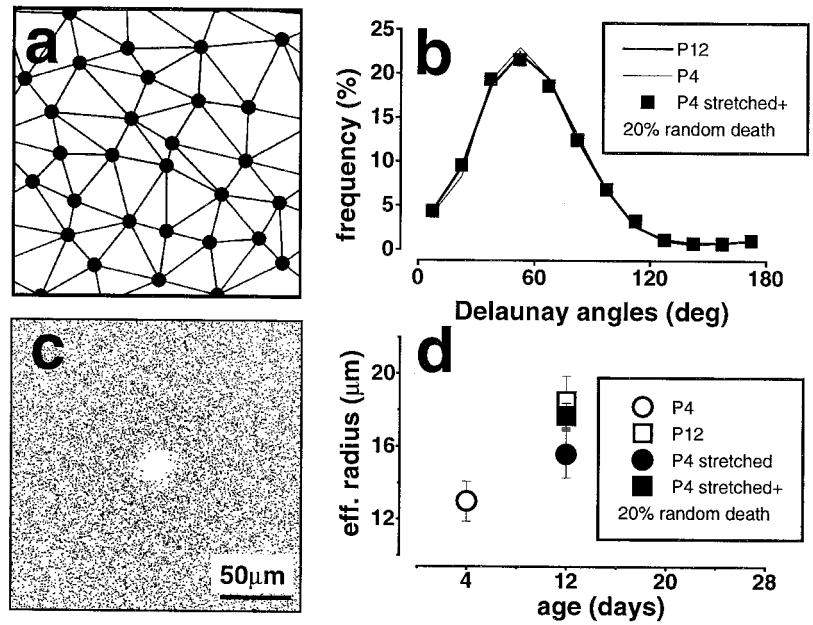

Figure 4. A random elimination of cells in the INL cholinergic array simulates the changes observed in the array spatial features between P4 and P12. *a,* An example of the Delaunay triangulation associated to an array. *Dots* represent array cells. The angles enclosed between contiguous Delaunay segments can be used to support the assumption of isotropic retinal growth. If anisotropic growth were to occur, the distribution of these angles should broaden and flatten, because the direction of major stretching contributes more acute angles and the direction of reduced expansion larger angles than the original distribution. *b,* The P12 angle distribution (*thick line*) is perfectly fitted by the angle distribution obtained, assuming retinal isotropic growth and 20% random cell elimination (*squares*). Note that the P4 array had already a very similar angle distribution (*thin line*). *c,* The autocorrelation of the cholinergic array cells is a uniform distribution with a central empty region. This reveals that the cholinergic arrays are organized as distributions based on an exclusion rule, by which no two array cells can be closer than a fixed minimal distance (Galli-Resta et al., 1997). An effective radius has been defined that gives a measure of the empty central region of the autocorrelation (Rodieck, 1991). *d,* The average effective radius obtained on P4 (*open circle*) is lower than that obtained on P12 (*open square*). Stretching the P4 array (*filled circle*) does not suffice to predict the P12 data, but adding the random elimination of 20% of the cells leads to an average effective radius that matches the P12 data (*filled square*).

(where the cell number is constant between P4 and P12; Fig. 1*b*). This assumption is further supported by the analysis of the angles between contiguous Delaunay segments (Fig. 4*a*). If retinal growth were anisotropic, the distribution of these angles should broaden and flatten, because the direction of major stretching contributes more acute angles and the direction of reduced expansion larger angles than the original distribution. Figure 4*b* shows how the P12 distribution of Delaunay angles (*thick line*) is well matched by the distribution obtained for simulations based on isotropic stretching (*squares*). Figure 2, *c* and *d*, shows the comparison between the P12 nearest neighbor distance and Delaunay segment distributions (*thick lines*) and the corresponding distributions obtained by simulating an isotropic expansion between P4 and P12 and the random loss of 20% of the cells (*squares*). The simulated distributions are very close to the real data, although they do not provide a perfect fit. This should not be surprising, considering that all simulations are based on average values for the retinal expansion factor and the amount of cell loss. To test further the agreement between the simulated and the real data, we analyzed the array autocorrelation. The developing arrays of cholinergic cells have been shown to be statistically equivalent to distributions where no two cells can be closer than a fixed minimal distance (Galli-Resta et al., 1997). When plotting the distances between any two cells in the array (autocorrelation), a central empty zone is obtained, as expected for distributions based on an exclusion rule (Fig. 4*c*). A measure of this empty region is the effective radius (Rodieck, 1991). Figure 4*d* shows the average effective radius (and its SD) obtained for the INL array on P4 and P12 (*open circle* and *square,* respectively), together with the same measure obtained from the simulations based on 20% random cell loss (*filled square*). This analysis confirms the good agreement between the P12 data and the simulations obtained assuming random cell loss.

DISCUSSION

We have shown here that the naturally occurring loss of 20% of the cells observed in the INL cholinergic array of the rat retina between P4 and P12 does not improve array regularity. Between P4 and P12 we did not observe any increase in the regularity index or in general any sharpening in the distribution of nearest neighbor distances and Delaunay segments. Computer simulations where the real mosaic fields are subject to isotropic stretching to simulate retinal growth and to the random elimination of 20% of their cells produce a good replica of the real data, suggesting that cell loss in this phase is largely independent of cell positioning. The temporal window chosen for this analysis corresponds to a period when the number of cells in the INL cholinergic array decreases and the addition of new cells to the array is likely to have come to an end. Indeed, the genesis of the cholinergic amacrine cells occurs prenatally (Reese and Colello, 1992) in the rat. By P4 (6 d after the end of genesis), all the cells are likely to have reached the INL, which is ~200 μm away from the region of new cell genesis [or 2 d away, considering a migration rate of 100 μm/d (Jacobson, 1991; Galli-Resta and Ensini, 1996)]. The same decrease in the number of cells in the INL array is observed, irrespective of the marker used to label the cholinergic cells (ChAT or Islet-1). Thus, cell loss rather than the cell failure to maintain the expression of a cholinergic marker is likely to account for these results. In addition, the constant number of ChAT cells in the GCL array suggests that the cholinergic cells missing from the INL did not move to the second array. Rather, death appears the prevalent or exclusive contribution to the variation in the number of cells in the INL array at these times.

Our analysis shows that death in this time window does not improve array regularity. Rather, death is indifferent to cell positioning. Yet, the regularity of the cell array is not drastically reduced, because it would be the case for example if the same percentage of elements were randomly eliminated in a crystalline array. This tolerance to the random elimination of a limited number of cells is a typical feature of cell distributions based on exclusion rules, which resist degradation better than arrays built on more rigid rules (Galli-Resta et al., 1997; Galli-Resta, 1998). These results differ from what reported for the α ganglion cell mosaics, where selective elimination of wrongly positioned cells has been shown to simulate array refinement (Jeyarasasingam et

al., 1998). However, this process leads to the segregation of the ON and OFF α ganglion cells from an initial common array of ON-OFF cells. This event has no direct parallel in the case of the cholinergic amacrine cells, which are segregated in ON (GCL) and OFF (INL) arrays by cell positioning into different layers (Masland and Tauchi, 1986; Voigt, 1986; Vaney 1990).

The independence of cell death from cell positioning suggests that death in the INL cholinergic array, at least in this period, might be related to processes other than the establishment of cell spatial ordering. Processes to which death has been classically linked, such as the establishment of synaptic connections and trophic interactions between input and target neurons, might control cell loss in this phase (Oppenheim, 1991). Indeed, the last born elements among retinal neurons, the bipolar cells that feed visual information to amacrine and ganglion cells, are appearing at these times (Cepko, 1993) when the first visual responses are also detected (O'Leary et al., 1986; L. Galli-Resta, unpublished observations). We cannot exclude, however, that death could be instrumental in establishing array order at earlier stages of mosaic assembly. Different approaches shall be used to investigate this issue before P4, when death occurs simultaneously to new cell addition (Galli-Resta and Ensini, 1996), making it difficult to asses the contribution of cell removal to array formation.

Between P4 and P12, cell death brings about a reduction of array regularity, albeit scarcely significant, and a twofold increase in the variability of the regularity index across the retina. This suggests that the regular spacing of cell bodies might be more crucial in the developing than in the adult retina. Indeed, the establishment of modular circuitry relies on synaptic connections that are made mostly on cell processes. Thus, functional circuits are likely to tolerate more variability in the positioning of the single cell bodies, once a sufficient dendritic tiling is achieved. On the contrary, the higher regularity of cholinergic cell positioning in the developing retina betrays the efficiency of the active processes that establishes regular cell positioning in the cholinergic arrays and maintains it throughout the period of new cell addition (Galli-Resta et al., 1997). The dynamic maintenance of array cell spacing during development may reflect the importance of positional constraints in controlling local cell density, tangential migration, and possibly earlier phases of cell death.

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