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Cell Death in the Mammalian Visual System During Normal Development: I. Retinal Ganglion Cells

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ABSTRACT

Degenerating cells may be observed with light microscopy in the hamster retinal ganglion cell layer during early postnatal development. On the first postnatal day, degenerating cell profiles were found at a rate of 2.7 per 1,000 live cells. This rate increased to a peak of 14.7 degenerating cells per 1,000 live on postnatal day 5 and then slowed to 4.2 per 1,000 live by postnatal day 10. These rates of cell death correspond to a 49% reduction in cell number in the ganglion cell layer. Examination of the spatial pattern of cell death revealed that although on visual inspection degenerating cells appear to occur in clumps, statistical analyses demonstrated a random distribution within retinal areas. Across the retina, cell death rates were higher in peripheral retina than in central retina. The timing and pattern observed correspond well with that of cell degeneration observed in the superficial layers of the superior colliculus, the major target of the retinal projection.

Normally occurring cell death has been found to be a characteristic common to developing vertebrate retinas. In the frog, periods of cell death were shown to coincide with the differentiation of the retinal layers (Glücksmann, '40). The impressive magnitude and orderly temporal and spatial patterns of cell death in the chick retina have been described in detail in several studies (Rager and Rager, '78; Hughes and McLoon, '79) and these patterns have been interpreted as contributing to the development of retinotopic projection. In chick, the role of the target fields in the regulation of retinal ganglion cell death has been demonstrated with an increased cell death following tectal lesions (Hughes and Lavelle, '75; Hughes and McLoon, '79), suggesting a dependence of these cells on central connections after some critical time.

In the rat, cell death is suggested to be important for the shaping of the optic fissure and may play a role in the outgrowth of ganglion cell axons (Silver and Hughes, '73). Except for the observation of a few degenerating cells in the inner nuclear layer of the rat retina on postnatal day 2 (Glücksmann, '51), little attention has been given to retinal cell death in mammals. The present study is an attempt to quantify and temporally and spatially describe cell death in the mammalian retinal ganglion cell layer, especially with regard to those features that it may share with retinal cell death in the chick. As these features have been considered in light of their role in the establishment of central connectivity in the chick, their presence in the mammalian retina may be an important factor for both normal development and plastic reorganization of the mammalian visual system. A preliminary report of this investigation has been published (Sengelaub and Finlay, '80).

METHODS

Subjects

Twenty-seven hamster pups *(Mesocricetus auratus)* from seven litters provided the retinas used in this study. Only animals delivered in the normal 15.5- to 16-day gestation period were used to match postconceptional ages. Three retinas each for postnatal days 1, 2, 3, 4, 5, 6, 7, 8, and 10 were examined, where postnatal day 1 is the day of birth.

Histology and counting procedures

Pups were taken from their mothers on the prescribed day, overdosed with urethane and perfused intracardially with a 4% formalin-45% alcohol solution. The head was removed, jaw and scalp dissected away, and the eyes left in place in the snout to retain proper orientation. Tissues were subsequently dehydrated with a series of alcohols, embedded in Paraplast (Lancer), cut horizontally at 10

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μm, and stained with cresylecht violet. One retina from each animal was selected (13 left, 14 right) from which one section each from the upper third, middle, and lower third of the retina was counted. Drawings were made of retinal cross sections at 500 × with locations and complete counts of all degenerating and normal cells in the ganglion cell layer, which is distinguishable by postnatal day 1.

Nuclei of degenerating cells undergo a series of characteristic changes, the earliest of which is the loss of internal detail. As degeneration continues, the nuclei shrink in volume and may subsequently fragment. These nuclei stain darkly and evenly, and have a liquidlike appearance (Silver and Hughes, '73; Hughes and McLoon '79; Chu-Wang and Oppenheim '78). These changes are apparent by light microscopy. Degenerating cells were thus counted after being identified by their darkly staining, shrunken, and sometimes fragmented nuclei, and their pale or absent cytoplasm (Fig. 1).

A normal cell was counted if it contained a bounded nucleus and at least one nucleolus; no attempt was made to distinguish neurons from glia. The ratio of degenerating to normal cells was then calculated after correcting for frequency of encounter by cell size and section thickness by the method of Abercrombie ('46).

RESULTS

Degenerating cells are present in the developing retina throughout the first 10 postnatal days (Figs. 1, 2, 3), during which the ganglion cell layer undergoes substantial changes in size and appearance. Degenerating cells are only occasionally seen on the first postnatal day in the presumptive ganglion cell layer as well as throughout the rest of the retina. At this point, the ganglion cell layer is eight to 12 cells wide and densely packed, being separated from the outer retinal layers by a narrow, poorly defined, cell-sparse transient zone. The outer retinal layers mostly consist of elongated and darkly staining cells which are packed more densely than the ganglion cell layer. The ganglion cell layer becomes more distinct in later days as the retina continues to stratify, especially with the formation of a definitive inner plexiform layer. The ganglion cell layer's width decreased during this time, becoming only one to two cells thick by postnatal day 10. The layer's length also changes dramatically, increasing in circumference 2.5 times by postnatal day 10. At the end of this period the ganglion cells are still immature, being small in size and retaining multiple nucleoli. The first Nissl material becomes apparent on days 7–10. Figure 1 shows representative sections through the developing ganglion cell layer for postnatal days 2, 5, and 10.

During postnatal days 1–3 degenerating cells were observed at a low and fairly constant rate in the ganglion cell layer ($\bar{x}$ = 2.58 per 1,000 live cells). This rate increased rapidly, peaking at a mean of 14.73 per 1,000 live cells on postnatal day 5 and then slowing gradually to a rate of 4.28 per 1,000 live cells by postnatal day 10 (Fig. 3). The variability in the cell death rate followed a similar trend in being low at first (S.E.M. = ±0.56), highest on postnatal day 5 (S.E.M. = ±2.36), and becoming low again (S.E.M. = ±0.61) by postnatal day 10.

Magnitude of cell death in the mammalian retina

Experimentally induced deviations in the amount of observed cell degeneration and the corresponding changes in cell density present after degeneration has ceased can be used to infer the magnitude of normal cell degeneration. Hughes and McLoon ('79) based their estimate of ganglion cell loss in the chick on the relationship of the retinal cell death rate and the resulting cell density in normal animals to that of animals who had received tectal ablations early in development. After a tectal ablation, cell death in the retina was greater than normal as reflected by (1) greater counts of degenerating cells in the experimental retinas, and (2) a lesser cell density after degeneration had stopped in the experimental retinas as compared to normals. By equating these two, Hughes and McLoon were able to obtain an estimate of 20% cell loss under normal conditions.

We have applied this relationship of differences between degenerating cell counts of normal and experimental retinas and the resulting differences in cell density to get an estimate of the cell loss in the hamster retina over development. When one eye is removed at birth in the hamster, the cell death in the remaining retina is reduced as reflected by the observation of fewer degenerating cells over the first 10 postnatal days and an increased cell density in the enucleated animals relative to normal (Sengelaub and Finlay, '81). Following the Hughes and McLoon method, normal cell death (CD_n) was determined using the relation of the decreased cell death rate in enucleates to that of normals (x = 0.923) and the differences between the mean cell density in normal and enucleate retinas ($\bar{x}_n$ = 157,000; $\bar{x}_e$ = 169,000) at adulthood (unpublished data, this laboratory):

$$CD_n = \frac{(\bar{x}_n - \bar{x}_e)}{x - 1}$$

Substituting the appropriate values yields a normal cell death (CD_n) of approximately 155,000 cells from the retina over the first 10 postnatal days. If the cell population present before cell degeneration is taken to be the cell density of normal adult retinas (x_n = 157,000) plus the normal cell loss (CD_n = 155,000), or a total of 312, 000 cells, then this represents a loss of 49% of the cells in the ganglion cell layer by cell death. As this method of estimating cell loss does not take cell death after postnatal day 10 into account, it is possible that our figure of 49% is an underestimate of the actual cell loss.

These calculations ignore addition of cells to the live cell pool in the early postnatal period. Although available evidence from other rodent species matched for postconceptional age indicates that all ganglion cells should be generated by the 16th postconceptional day (Bruckner et al., '76; Morest, '70), substantial glia are added postnatally. Glial addition would diminish the apparent cell death rate, resulting in an underestimation of the amount of death in the original neuronal population. For example, if 10% of the neurons are actually being lost on a particular day from a population of 1,000, and 500 glia are added in the same day, the observed degenerating cell/normal cell ratio will be 6.4%. Similarly, the addition of 100 glia cells would give an apparent ratio of 9%. Since we do not yet know rates and amounts of glial addition in the hamster retina during the early postnatal period, we will only assume our calculations somewhat underestimate the actual neuron loss.

Although glial degeneration has not been described, the possibility of glial cell death might further influence our calculations. As stated above, addition of glia postnatally could result in an underestimation of cell loss. This under-

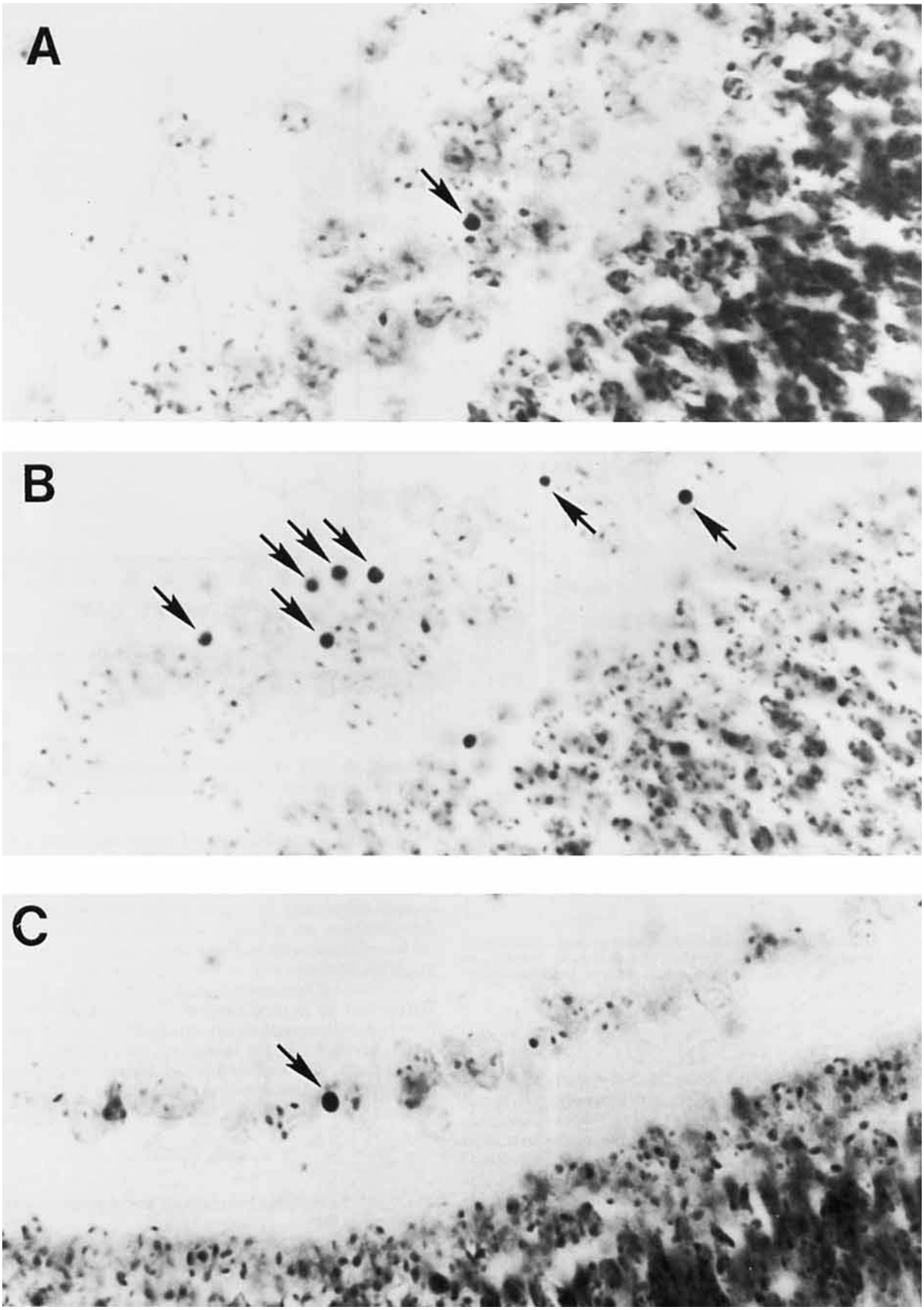

Fig. 1. Photomicrographs of horizontal sections through the developing hamster retina at postnatal day 1 (A), day 5 (B), and day 10 (C). Arrows indicate degenerating cells in the ganglion cell layer. cresylecht violet stain, × 400.

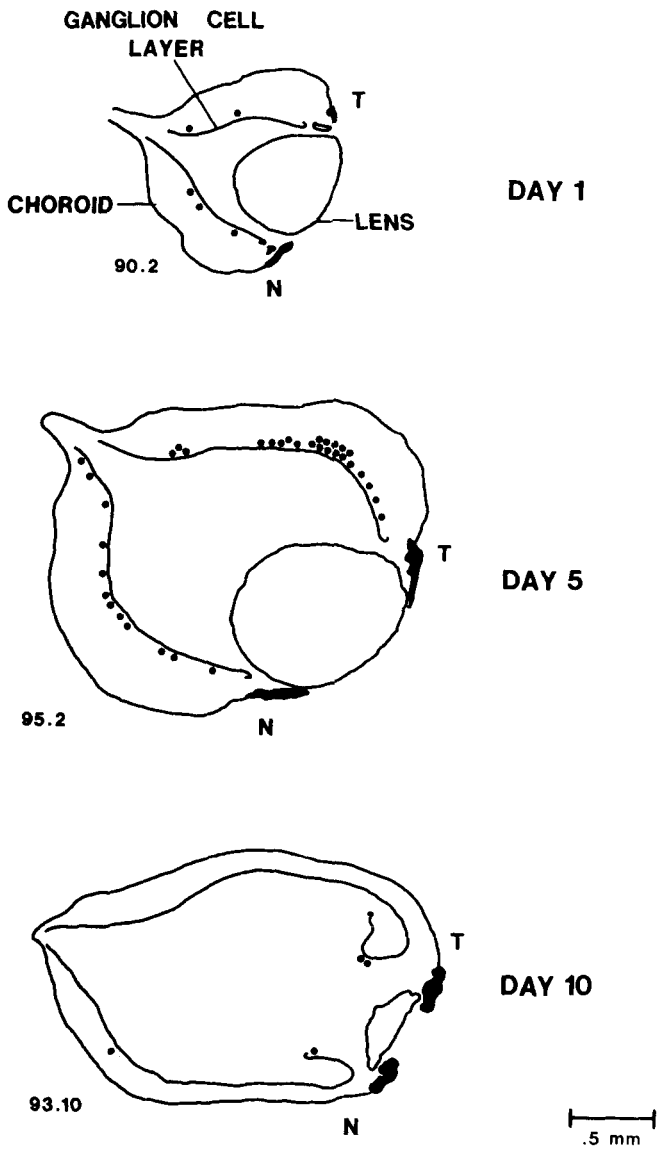

Fig. 2. Drawings of representative retinal cross sections at the level of the optic disc for postnatal days 1, 5, and 10. Dots indicate the number and location of degenerating cells in the ganglion cell layer. Abbreviations: N, nasal; T, temporal.

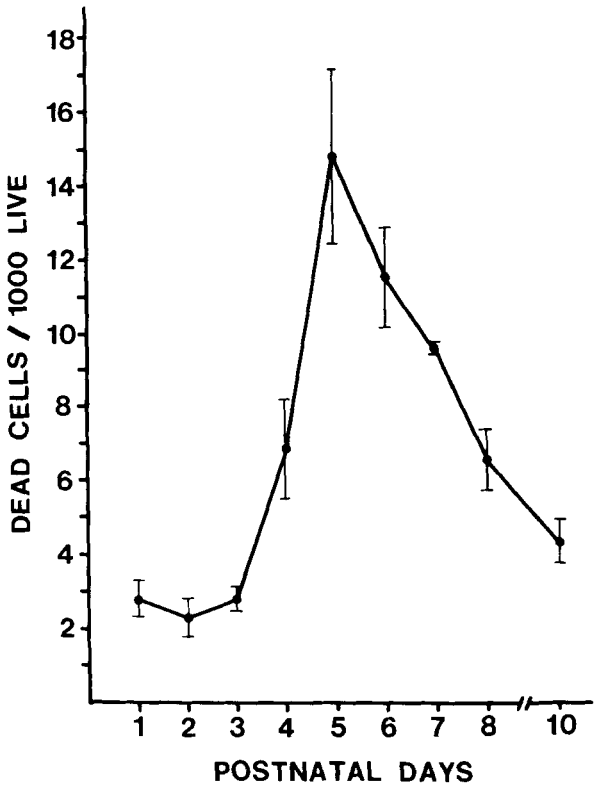

Fig. 3. Ratio of degenerating cells to normal cells in the ganglion cell layer of the hamster retina over the first 10 postnatal days. Points represent averaged values ± standard error of the mean.

estimation would likewise occur if there were no degeneration in a glial cell population. For example, if in a cell population that is comprised of equal numbers of neurons and glia, 10% of the neurons are actually being lost, then the observed degeneration rate would be only 5%. Similarly, if glial degeneration were occurring at any rate less than that of neuronal degeneration, an underestimation of neuron loss would result. Equal degeneration rates in both neurons and glia would, of course, have no effect on the apparent rates because the same percentage would be lost from the total cell population. Finally, glial degeneration at rates higher than the neuronal degeneration rates would yield overestimates in the apparent cell degeneration. Glia are present in approximately a 1:1 ratio with ganglion cells in the adult hamster retina (unpublished observation).

Spatial distribution of degenerating cells: clumping

Since the distribution of degenerating cells with respect to each other may be a clue to the function of this early degeneration, an extensive analysis of the spatial distribution of these cells in the retina was done. The nearest-neighbor analysis of Clark and Evans ('54, '79) was used to determine if degenerating cells were clumped, randomly distributed, or hyperdispersed. This measure is the ratio $R = r_o/r_e$, where r_o is the mean observed nearest-neighbor distance, while r_e is the mean expected value for a random distribution. The significance of departures from the expected value is tested using the formula:

$$C = \frac{\bar{r}_o - \bar{r}_e}{\sigma r_e}$$

where c is the standard variate for the normal curve (Clark and Evans, '54).

Since the expected values are determined by the density of cells (Clark and Evans, '79) and cell density was not uniform along the retinal cross sections, each retinal cross section was divided into six equal segments, and the linear cell density for that segment was computed. The observed

interdegenerating cell distances were then weighted by the linear density estimate at the midpoint of each intercell distance.

The nearest-neighbor measures (R) obtained from postnatal days 4 to 7 (three animals/day, three measurements/animal), the days of maximally observable cell death, ranged from 0.3 to 1.44 with a mean of 0.92 (Fig. 4). In no case was a departure from r = 1.0, which indicates a random distribution, found to be significant. The standard variate values ranged from −0.89 to +0.96, where c = 1.96 would be significant at the 0.05 level. This is in spite of the large number of tests performed (36) which would increase the likelihood of observing extreme values by chance alone. Thus, we are unable to reject the hypothesis that the degenerating cells are randomly distributed.

Nevertheless, on visual inspection, degenerating cells in the retina do appear to be clumped. This visual impression does not take into account changing variations in cell density. To test if this impression was accurate, the same nearest-neighbor test was run on two retinas, three sections each, chosen for maximum subjective "clumpedness" without adjusting interdegenerating cell distances for density. The effect in each case was to shift the R ratio downward from 1.0 (average R = 0.815) toward the clumped distribution, but the largest standard variate observed was c = 0.67, well below the 0.05 significance level of 1.96.

Spatial distribution of degenerating cells: cross-retinal distribution

A second feature of interest in the spatial organization of degenerating cells is their changing distribution over time across the retinal surface. In chick, Rager and Rager ('78, '80) have reported a central to peripheral progression of maximum observable cell degeneration, corresponding to the pattern of retinal maturation, while Hughes and McLoon ('79) report a temporal to nasal progression, which they related to the pattern of midbrain innervation by retina. We investigated hamster retinas for both of these patterns.

Retinal cross sections were divided into their nasal and temporal halves, and the cell death rate per retinal half was calculated. Both temporal and nasal retina peaked on day 5, and no striking precedence in magnitude of cell death by retinal half was observed. There were no statistically significant effects.

Retinal sections were also divided into central and peripheral divisions by bisecting the horizontal and vertical radii of the reconstructed retinae. Each counted section was then parsed into its central and peripheral components. Although the cell death rates for both central and peripheral retina peaked on the same postnatal day, day 5, higher rates were found centrally at the onset of cell death and higher peripherally as it subsided (Fig. 5). Interestingly, the cell death rate in peripheral retina was higher overall, and approached statistical significance [$F(1,36) = 3.12$, $0.05 < P < 0.1$].

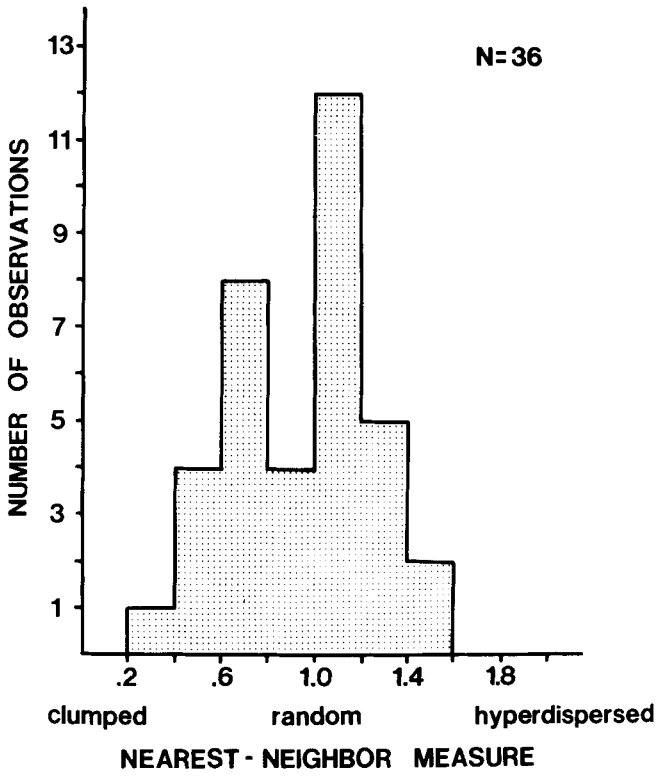

Fig. 4. Frequency histogram of nearest-neighbor values (R) obtained from the spatial analysis of degenerating cells in retinal cross sections. R values of 1 indicate a random distribution of degenerating cells; greater R values indicate a hyperdispersed distribution; lesser R values denote a clumped distribution. No distribution of degenerating cells was found to differ significantly from random.

DISCUSSION

Magnitude of cell death

We have made a conservative estimate of the magnitude of cell death in the hamster retina by neglecting prenatal and post-day 10 cell death as well as the effect of postnatal glial addition. This estimate gives a 49% cell loss in the first 10 postnatal days. Although this estimate ignores the possibility of a differential cell degeneration between neuronal and glial populations, this question could be addressed through the use of thymidine autoradiography or immunocytochemical markers in order to selectively identify neurons and glia and assess changes in these populations separately during the period of cell death. Regardless of these limitations, the cell loss estimation shows that the apparently low rate of degeneration can correspond to a substantial cell loss and gives a general sense of the order of magnitude of cell death in the mammalian retina.

Other estimates of amount of cell death in spinal cord and various cranial nerve nuclei range from 40 to 75% (Cowan, '73). For chick retina, Hughes and McLoon ('79) report a ganglion cell loss of 20% during normal development, while Rager and Rager ('78) report a 40% decrease in the number of optic nerve fibers during development. Thus, our estimates of the magnitude of cell death in the mammalian retina correspond well to the range reported for other vertebrates.

Significance of the spatial distribution of degenerating cells

Several investigators have noticed in their surveys of degenerating cells that their distribution appears to be patchy or clumped, in tectum (Arees and Astrom, '77; Berg

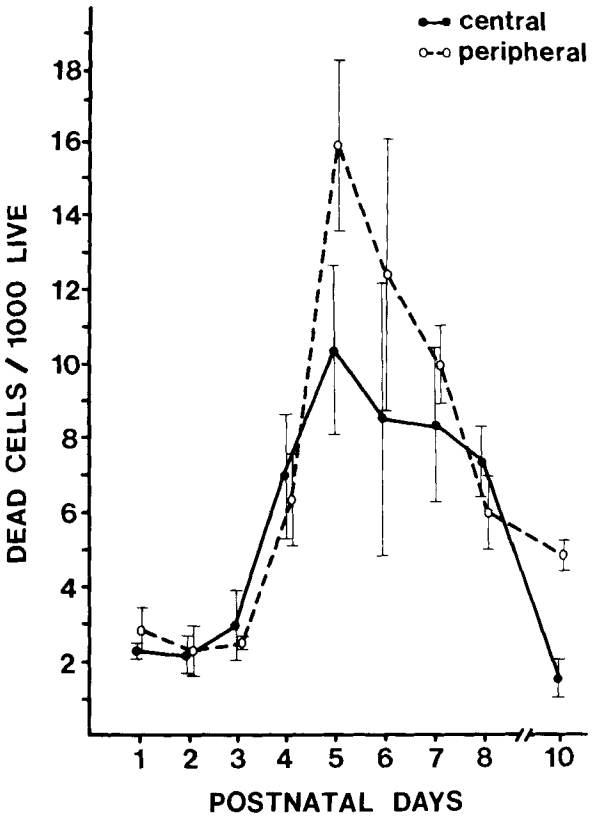

Fig. 5. Ratio of degenerating cells to normal cells in the ganglion cell layer over the first 10 postnatal days for central (solid lines) and peripheral (broken lines) retina. Points represent averaged values ± standard error of the mean. While both central and peripheral cell degeneration rates peak on postnatal day 5, cell degeneration is higher in peripheral retina.

and Finlay, '79; Giordano and Cunningham, '78) and in retina (Hughes and McLoon, '79). Close spatial association of degenerating cells, if the case, suggests a variety of hypotheses for the cause: induced death by a local metabolic accident; a clonal relationship of the degenerating cells; or induced degeneration of synaptically associated cells. However, our nearest-neighbor analysis of the pattern of cell death revealed no significant deviations from a random distribution. This result argues for more caution in the description of population distributions: specifically, it argues that human observers tend to view a population that is in fact hyperdispersed as random, and a random distribution as clumpy or patched.

We found no evidence for a temporal to nasal progression of cell death in hamster as was seen by Hughes and McLoon in the chick, even though the pattern of tectal generation and innervation is roughly comparable between the two animals. We found some evidence for a central to peripheral change over days in observable cell degeneration, in line with the general central to peripheral pattern of neurogenesis in the rodent (Morest, '70). The finding of elevation of cell death in the peripheral retina relative to central retina may be related to a corresponding pattern of cell degeneration in the tectum (Finlay, Berg, and Sengelaub, '82), and raises the very intriguing possibility that some local specializations of retina might be partially sculpted by differential cell death.

Cross-species comparisons of cell death during neurogenesis

We have investigated a variety of aspects of cell death in the rodent retina that may be compared to what is known for chick retina and nervous system: appearance of degenerating cells; correlation of maximal degeneration and maturational state of surrounding cells; magnitude of degeneration; spacial and temperal distribution of degenerating cells. The similarities are striking. Maximal degeneration appears to correlate with synapse formation at principal retinal targets (Frost et al., '79; Cantino and Sisto-Daneo, '72) but preceeds mature cytodifferentiation. The order of magnitude appears to be comparable. Species differences in the pattern of retinal maturation are reflected in the cross-retinal patterns of cell death. Of course, these observations are not in the least remarkable in that substantial neuron death appears to be a major feature of vertebrate neurogenesis. Until quite recently, however (Cunningham et al., '79; Sengelaub and Finlay, '81), cell death has been substantially ignored as a central feature of visual system development in mammals, both in normal development and in plastic reorganization of mammalian visual system consequent to early damage. A variety of features of early visual system development in mammals such as removal of early exuberant connectivity, sequestration of contralateral and ipsilateral retina projection zones, and alteration of connectivity patterns consequent to early lesions should be reconsidered in light of this substantial normally occurring degeneration in development.

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