Timing and Topography of Cell Genesis in the Rat Retina

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ABSTRACT

To understand the mechanisms of cell fate determination in the vertebrate retina, the time course of the generation of the major cell types needs to be established. This will help define and interpret patterns of gene expression, waves of differentiation, timing and extent of competence, and many of the other developmental processes involved in fate acquisition. A thorough retinal cell “birthdating” study has not been performed for the laboratory rat, even though it is the species of choice for many contemporary developmental studies of the vertebrate retina. We investigated the timing and spatial pattern of cell genesis using ³H-thymidine (³H-TdR). A single injection of ³H-TdR was administered to pregnant rats or rat pups between embryonic day (E) 8 and postnatal day (P) 13. The offspring of prenatally injected rats were delivered and all animals survived to maturity. Labeled cells were visualized by autoradiography of retinal sections. Rat retinal cell genesis commenced around E10, 50% of cells were born by approximately P1, and retinogenesis was complete near P12. The first postmitotic cells were found in the retinal ganglion cell layer and were 9–15 μm in diameter. This range includes small to medium diameter retinal ganglion cells and large displaced amacrine cells. The sequence of cell genesis was established by determining the age at which 5, 50, and 95% of the total population of cells of each phenotype became postmitotic. With few exceptions, the cell types reached these developmental landmarks in the following order: retinal ganglion cells, horizontal cells, cones, amacrine cells, rods, bipolar cells, and Müller glia. For each type, the first cells generated were located in the central retina and the last cells in the peripheral retina. Within the sequence of cell genesis, two or three phases could be detected based on differences in timing, kinetics, and topographic gradients of cell production. Our results show that retinal cells in the rat are generated in a sequence similar to that of the primate retina, in which retinogenesis spans more than 100 days. To the extent that sequences reflect underlying mechanisms of cell fate determination, they appear to be conserved. J. Comp. Neurol. 474:304–324, 2004. © 2004 Wiley-Liss, Inc.

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The mechanism of cell fate determination in the nervous system is an important and active area of investigation, and for many reasons the retina is probably the most widely studied tissue. From early studies, two significant observations have emerged: 1) all retinal cell types can be generated from a single progenitor (Turner and Cepko, 1987; Holt et al., 1988; Wets and Fraser, 1988; Turner et al., 1990), although 2) not all cell phenotypes are generated at the same time. A temporal order to the genesis of different cell phenotypes is suggested by the latter finding (Sidman, 1961; Young, 1985a; Harman and Beazley, 1989; LaVail et al., 1991). Since these observations, research has concentrated on identifying the molecular and cellular cues that operate within this framework to determine cell fate. For example, a number of genes are known to be expressed during various stages of retinal development. For some, interfering with expression in either a positive (overexpression, expression of an activated construct) or negative (gene knockout, expres-
tion of dominant-negative construct, expression of antisense construct) can influence the cellular composition of cultured retinal cells or in vivo retinas (for recent reviews, see Perron and Harris, 2000; Livsey and Copko, 2001; Vetter and Brown, 2001; Marquardt and Gruss, 2002). Given that cells are generated according to a timetable, it is important to know for any organism the schedule of production of different types. For example, to test whether a gene is involved in rod photoreceptor fate acquisition using a rat retinal cell culture paradigm (as in Levine et al., 1997), the time of genesis of rat rods needs to be known.

Most studies of molecular interactions during retinal development are performed on standard laboratory species, for mammals typically strains of rat and mouse. Rodents are used because they are small, easily housed and fed, breed readily, have a short gestation time, and mature rapidly. In addition, recently developed genetic methods offer the opportunity to alter gene function. However, the fact that the rat and mouse develop rapidly has a significant cost—the temporal resolution of developmental events can be poor. Nevertheless, one of the first attempts to establish the timing and order of cell genesis in the nervous system was performed by Sidman (1961) in the mouse retina. Although groundbreaking in a number of ways, this early study was incomplete because the histological methods used could not distinguish individual cell types within retinal layers. Subsequent research has further addressed the question of retinal genesis in rodents. Based on the extant literature, we know that in the mouse and rat, retinal ganglion cells are among the first cells generated (Sidman, 1961; Reese and Colello, 1992; Galli-Resta and Ensini, 1996). Amacrine cells with different transmitters are generated during different periods from mid-prenatal to early postnatal stages (Evans and Battelle, 1987; Zhang and Yeh, 1990; Reese and Colello, 1992; Lee et al., 1999). Cone photoreceptors are generated early, entirely prenatally (Carter-Dawson and LaVail, 1979b), whereas rods are generated late, and significant numbers are generated postnatally (Carter-Dawson and LaVail, 1979b; Ila and Jeffery, 2000). Finally, the primary cell types generated postnatally are rod photoreceptors, bipolar cells, and Muller glia (Blanks and Bok, 1977; Young, 1985a). None of these studies provides a comprehensive quantitative analysis of the timing, spatial pattern, and kinetics of genesis of all seven cell types found in a rodent retina.

A thorough analysis of cell genesis must satisfy three criteria. First, it must define the limits of cell production by starting with ages before any cells are generated and ending after all cells have been formed. Second, it must sample many ages during the entire period between initiation and termination of cell genesis. Third, it must be able to recognize cell types and consider them all. Although, as we have noted, a literature already exists on retinal cell genesis in the rodent, including rat, and these studies have contributed pieces of the puzzle, none satisfy all three of these criteria. For example, several studies of rodent retinal cell genesis focused on the postnatal period only (Blanks and Bok, 1977; Young, 1985a). Others were unable to recognize different phenotypes (Sidman, 1961; Gloor et al., 1985; Ila and Jeffery, 2000). Finally, many papers have considered the genesis of only specific cell types, including: retinal ganglion cells (Draeger, 1985; Reese and Colello, 1992; Galli-Resta and Ensini, 1996; Ila and Jeffery, 1996; Rachel et al., 2002), amacrine cells (Evans and Battelle, 1987; Zhang and Yeh, 1990; Lee et al., 1999), and photoreceptors (Carter-Dawson and LaVail, 1979b; Ila and Jeffery, 2000). Of these, only a few specified the ages at which genesis of each type begins or ends. For example, the study of ganglion cell genesis is of particular interest because these cells are among the earliest types generated and therefore may specify the initiation of overall retinal cell production. However, of the studies looking at ganglion cell genesis, only one (Reese and Colello, 1992) determined a time point when no cells were labeled with a cell birthday marker.

Based on the experience we gained in determining retinal cell genesis in monkey (LaVail et al., 1991; Rapaport et al., 1992, 1995, 1996), we undertook a similar parametric study of the rodent retina. We performed a complete analysis of the temporal and spatial patterns of cell genesis, from the beginning to the end of cell production, and used histological techniques that allowed resolution of cytoplasmic and nuclear morphology to determine cell phenotype with accuracy. Availability of these data for the laboratory rat will aid in directing research on the molecular interactions leading to cell fate acquisition in the mammalian retina and the formulation of models that place specific regulatory molecules in the broader context of making the right cell types at the right time in the right number to form functional retinal circuits.

**MATERIALS AND METHODS**

**Surgery and histological preparation**

All animal experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the guidelines of the Committee on Animal Research at the University of California, San Francisco. Two congenic strains of inbred Royal College of Surgeons (RCS) rats, RCS-reddry− (pink-eyed, normal) and RCS-reddry+p (pigmented, normal), with normal retinas (LaVail, 1981) were used. Fertile male and female pairs were housed in hanging cages. The litter and bedding in the cage, as well as the litter and bedding that had fallen through the cage floor, were examined each morning and evening for the presence of a dislodged vaginal plug. When a plug was discovered, the animals were separated and placed in conventional cages, and the day was designated as E0 of a resulting pregnancy. The accuracy of the timing of our matings was verified by counting backward from the day of delivery, which was typically 22 days from the presence of a vaginal plug.

Pregnant dams or postnatal rat pups were given an intraperitoneal injection of 5H-methyl thymidine (5μCi/g, 6.7 Ci/m mole; New England Nuclear, Boston, MA). Animals were allowed to survive for 50–303 postnatal days, with the majority living for around 220 days. Even at the shortest survival time (P50) the retina is fully mature, and because the data seemed to fit regardless of whether the postnatal survival was short or long, we concluded that this was not a significant confounding factor. We processed and analyzed up to three animals for each injected embryonic and postnatal age from E8 to P13. The precise number of individuals at each injection age depended on the size of the litters and our need to process both radial and en face sections (see below). A total of 50 5H-TdR-injected rats were used.

The animals were killed by carbon dioxide anoxia followed immediately by vascular perfusion with buffered
mixed aldehydes (2% paraformaldehyde, 2.5% glutaraldehyde). After perfusion, the superior margin of each eye was marked, and then both eyes were removed and processed in one of two ways. Most eyes were bisected through the optic nerve head, from superior to inferior, approximately along the vertical plane, as previously described (LaVail and Battelle, 1975). These hemispheres were dehydrated in a 1:1 mixture of methanol and methyl cellosolve and embedded in glycol-methacrylate resin (GMA); then 1.5-μm-thick sections were cut. Sections were mounted on a microscope slide and air-dried. In another set of eyes, the anterior portion and sclera were removed, and the remaining retina and choroid were flattened onto a glass microscope slide by making several radial cuts around the perimeter (Stone, 1981). To keep the retina flat during dehydration, the tissue was covered by a sheet of weighing paper that was held to the slide with paper clips (this method was shown to us by A. Hendrickson). This “sandwich” was dehydrated as above, and immediately prior to embedding the clips were removed and the retina freed from the slide. The tissue was embedded in polyester wax, and serial sections were cut at 10 μm, en face, from the vitreal surface. These slides were dewaxed in xylene, rehydrated in a descending series of alcohols, and air-dried.

Dry slides containing the radial and en face sections were dipped in Kodak NTB-2 photographic emulsion and stored in the dark at 4°C for 4–16 weeks. The appropriate exposure time was determined by weekly development of test sections. When a common maximal label (18–20 grains/nucleus) was deemed to be present, the entire series was developed in Kodak D-19 and counterstained with 0.125% toluidine blue (Rakic, 1973). Selected images of the slides were acquired with an Apogee KX85 CCD digital camera and saved as grayscale Adobe Photoshop files. The brightness and contrast were optimized, and images were cropped to the size needed to construct the montages in Figures 1 and 2.

Determining labeled cells

A critical parameter in autoradiographic studies of cell birth is the density of exposed silver grains over a nucleus that is adopted as the criterion for “heavy” label (Fig. 1). The density of the radiographic signal is primarily a reflection of the number of cell divisions since the 3H-TdR incorporation. It is well established that the mode and route of administration provides a short pulse of availability. It is well established that the mode and route of administration provides a short pulse of availability of 3H-TdR, on the order of 30 minutes (Skougaard and Denham, 1967; Young, 1985b; Alexiades and Cepko, 1974; Hickey et al., 1983). The period of availability of 3H-TdR is short relative to the 8–10-hour cell cycle time (Denham, 1967; Young, 1985b; Alexiades and Cepko, 1996). The cohort of labeled cells can be said to have spent all or some of the window of 3H-TdR availability in S-phase. Given the length of S-phase and the speed of thymidine incorporation (Hayes and Nowakowski, 2000), only a minority of cells would have low grain counts due to leaving S-phase just after the 3H-TdR injection, or entering S-phase just as the pulse ended. The obligatory M-phase means that a cell that left the cell cycle after being exposed to 3H-TdR would have, on average, 50% of the radioactive nucleotide that was originally incorporated. This would decrease by approximately 50% with each further mitosis. A number of technical factors such as section thickness, emulsion thickness (which can vary even on a single microscope slide), and length of exposure of the autoradiograph also contribute to variation in grain counts. However, these sources of error were kept in control by adopting a standard maximum label density across all specimens.

We developed test sections on successive weeks (up to 12 weeks) until the maximum label density reached about 18–20 grains, at which point we developed the entire series. This grain density allowed enough dynamic range to distinguish heavy from background labeling readily, while not obscuring the cytological features on which we recognized different cell types (Fig. 2). A potentially confounding factor that we did not address was the influence of the size of a cell's nucleus on autoradiographic grain density. Cells born on a certain day should not differ in their absolute isotope content, but those with larger nuclei would have a lower isotope concentration. Carter-Dawson and LaVail, 1979b). We felt justified in taking this short cut because a criterion set for small cells would be conservative for large ones and would potentially lead to false negatives. We are less concerned about false negatives than false positives, and we cannot envision a mechanism, which we have not controlled for, that could lead to false positives.

In his pioneering study of cerebellar cell birthdays, Rakic (1973) set the threshold for a cell to be heavily labeled at 50% of the maximum. Although admittedly somewhat arbitrary, this criterion has been widely adopted by workers studying cell birthdays in many other brain regions, including the retina. To be able to compare the data contained herein with most of the prior studies, we have also used the 50% maximum criterion for determining that a cell has been “born.” Any cell with less than 50% of the maximum grain count, but that appeared to be labeled above background, was considered lightly labeled. Although we collected data on lightly labeled cells, they were not analyzed extensively. Nonetheless, they offered support to the analysis of heavily labeled cells and allowed us to extend it as necessary. The first 3H-TdR injection to produce heavily labeled cells was given at E10, and Figure 1 shows the heaviest labeled example of each cell type in one section. Three types (retinal ganglion cells [Fig. 1C,D], horizontal cells [Fig. 1G,H], and cones [Fig. 1K,L]) had a grain density exceeding the 50% of maximum criterion, so these cells underwent their final mitosis shortly after the E10 3H-TdR injection. The density of silver grains in these cells can be contrasted to lightly labeled profiles of the same types (Fig. 1A,B, E,F, and I,J, respectively). The most densely labeled amacrine cell (Fig. 1M,N), bipolar cell (Fig. 1O,P), rod (Fig. 1Q,R), and Müller cell (Fig. 1S,T) never reached the “heavy label” criterion. Indeed, it is questionable whether the Müller cell with its three grains (Fig. 1S,T) was even labeled above background.

Scoring, plotting and measuring labeled cells

We collected and analyzed data from as many different individual subjects as possible. If at least three rats from an injection cohort developed into adulthood, one section from each was included in the final analysis (n = 6 injection ages). If only two individuals were available, two sections from one animal and one section from the other (determined randomly) were studied (n = 11 injection
ages), and if only one animal was available, all three sections were from that individual (n = 10 injection ages).

We took care to ensure that the sections analyzed from different individuals were from corresponding retinal locations. If more than one section from an individual was used, the sections were separated by at least 9 μm. The procedure for scoring labeled cells was developed previously (see Fig. 1 of LaVail et al., 1991). Beginning at the optic nerve head, the retina was tracked in the superior (dorsal) direction by movement of the microscope stage in

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Fig. 1. Photomicrographs of labeled cells in the retina of a P54 rat exposed to 3H-TdR at E10. The vitreous is up and the sclera is down. Note the low level of background signal. Injection at E10 produced the first heavily labeled cells. A single section was carefully scanned, and the most heavily and most lightly labeled examples of each cell type were photographed to demonstrate the labeling density and how the heavily labeled cells stood out. Each cell type is shown as a pair (arrow)—with the focal plane adjusted on the tissue (cell nucleus), or on the exposed grains (grains) in the photographic emulsion overlying it. The most densely labeled cell was a retinal ganglion cell with 18 grains (C,D). Therefore, any cell on this slide with more than 9 grains was regarded as heavily labeled. All heavily labeled cells are set off by a black background and were one of three types: retinal ganglion cells (C,D), horizontal cells (G,H), or cone photoreceptors (K,L). Cells with less than nine grains, which were labeled above background, were considered lightly labeled. Examples of these are shown: ganglion cells (A,B), horizontal cells (E,F), cones (I,J), amacrine cells (M,N), and rod photoreceptors (Q,R). Bipolar cells (O,P) and Müller cells (S,T) exhibited a grain density that was only slightly above background, if at all. Abbreviations: RGC, retinal ganglion cell; Ho, horizontal cell; CPr, cone photoreceptor; Am, amacrine cell; BP, bipolar cell; RPr, rod photoreceptor; Mü, Müller glial cell. Scale bar = 10 μm in A (applies to A–T).
440-μm increments. This corresponds to the diameter of the microscope field at 400× magnification. For each field, lightly and heavily labeled cells were counted, and their identity was determined by reference to their laminar position, size, shape, and staining characteristics (Fig. 2 and see below). Once the superior ora serrata was reached, the stage was moved back to the optic nerve head, and the retina was tracked in the opposite direction until the inferior ora was reached. We assessed the density of genesis in any specific region of the retina and gradients of genesis among different regions by summing the number of labeled neurons in each 440-μm field and representing each field as a “bin” on histograms (Figs. 4, 8, 9).

Because there are substantial differences in retinal development related to retinal location (Rapaport and Stone, 1984), comparisons between central and peripheral regions were made. A central sample was defined as the central two-fourths of the entire linear extent of the retinal sections, one-fourth on either side of the nerve head. The peripheral sample was made up of each flanking quarter of linear extent. Thus, a section that spanned 20 bins (i.e., ~8.8 mm from superior to inferior ora) would be divided into a central zone consisting of five bins above and five bins below the optic nerve head (ONH), as well as a peripheral zone of the flanking five bins each in the superior and inferior retina.

Soma size was measured from 10-μm-thick en face sections through the ganglion cell layer (GCL). All data were collected from corresponding retinal regions 1 or 3 mm dorsal to the optic nerve head (the central and peripheral samples, respectively; the distance from the ONH to the ora serrata was typically 4 mm). The outlines of all cells in a field with defined nuclei and a dark nucleolus were drawn with a camera lucida until a sample of at least 100 was obtained. Each profile was identified as unlabeled, lightly labeled, or heavily labeled. These outlines were traced by using a computer graphics tablet, and the cell area was determined with Bioquant (Nashville, TN) software. Cell diameter was taken as that of a circle with the known area. Because all animals were mature at the stage of analysis, the heavy, light, and unlabeled diameter data from all individuals were pooled to provide a large, control sample of 2,097 cells. This “total population” (TP) contained retinal ganglion cells and displaced amacrine cells but not astroglia, microglia, or endothelial cells, which could be readily recognized based on shape and staining properties.

Recognition of cell phenotype

The classification of cell phenotype was based on laminar and intralaminar location as well as nuclear and cytoplasmic morphology, as illustrated in Figure 2. The features distinguishing different cell types are readily apparent in 1.5-μm sections stained for Nissl substance (Fig. 1). The criteria worked out by us for the monkey retina, which have been similarly developed for the rabbit (Strettoi and Masland, 1995), proved to translate readily to the rat. Cells in the GCL ranged from 5 to 22 μm in diameter (Fukuda, 1977; Perry, 1981; Dreher et al., 1985; and see Fig. 6), and had well-defined nuclei, prominent nucleoli, and Nissl substance in their cytoplasm. The largest (15–22 μm) are exclusively retinal ganglion cells (Perry, 1981; Huxlin and Goodchild, 1997), whereas the smallest (5–7 μm) are exclusively displaced amacrine cells (Perry, 1981; Mitrofanis et al., 1988; Mitrofanis, 1989; Kim et al., 2000; Oh et al., 2002). In Figure 2B the profile on the right is of a size to be reliably considered a retinal ganglion cell, whereas that on the left is either a small retinal ganglion cell or a displaced amacrine cell. For the most part, we did not distinguish neuronal profiles in the GCL separately as ganglion cells or displaced amacrine cells, although we make some conclusions in the Discussion based on population soma size data. Amacrine cells (Fig. 2C) line the inner margin of the INL, forming a discontinuous sublamina one to two cells wide, although cells with similar morphology were occasionally found deeper in the INL. Amacrine cells have large, lightly staining nuclei, prominent nucleoli, and usually a thin ring of pale cytoplasm around the nucleus.

Müller cells (Fig. 2C) are readily recognized by their scalloped, irregular outline, homogenous, darkly stained nuclei, and lack of obvious cytoplasm. For the most part they are located just to the outer side of the amacrine cell sublamina. Bipolar cells (Fig. 2C) form most of the outer half of the INL, although they can occasionally be present in the inner half. They have round somata, generally smaller than that of amacrine cells, medium to dark staining nuclei, and a ring of cytoplasm usually darker than that of amacrine cells. Nucleoli are seldom present. Horizontal cells (Fig. 2C) are sparsely distributed along the outermost margin of the INL. They have a fusiform shape oriented parallel to the retinal layers, a large, medium-staining nucleus, prominent nucleolus, and significant pale cytoplasm surrounding the nucleus. The majority of ONL profiles (Fig. 2D) are small and spherical, with uniformly darkly stained heterochromatin and almost no euchromatin. These are features characteristic of rods (Carter-Dawson and LaVail, 1979b). Scattered throughout the ONL (Fig. 2D), with a tendency toward placement in the outer half, are larger, oval profiles, with several clumps of heterochromatin surrounded by euchromatin, features that characterize them as cones.

RESULTS

Initiation, cessation, and rate of retinal cell genesis

Injection of 3H-TdR at E8 and E9 labeled some cells, but the density of label, although above background, was not sufficient for any to be considered “heavily” labeled. In addition, the E9 injected specimens tended to show a higher overall labeling density than E8 (data not shown). This indicates that the injected thymidine reached the retina, that progenitors went through several cell divisions diluting the signal before generating postmitotic daughters, and that the dilution was greater from E8 than E9. By E10, there were occasional heavily labeled cells, on average 20 per section, examples of which are shown in Figure 1. In the overall context of retinogenesis, the number of labeled cells is small; however, it suggests the age that cell birth begins. An important caveat is that, because we are analyzing mature retina some cells labeled by 3H-TdR injection may have subsequently been lost to naturally occurring cell death, a prominent feature of retinal development (for example, see Young, 1984; Horsburgh and Sefton, 1987; Robinson, 1988; Cook et al., 1998). It must be kept in mind that we are determining the birthdays of only those cells that survived.
Fig. 2. Photomicrographs from a 1.5-μm-thick section of a glycol methacrylate-embedded adult rat retina stained with toluidine blue. The region sampled is the superior retina at a middle eccentricity close to the border between what we define as central and peripheral zones, i.e., close to the junction of the superior quarter and middle two quarters of linear extent. The thin sections and optimum stain readily allowed recognition of cell phenotypes, and at least two examples of each are shown. A lower power photo (A) shows the normal retinal cytoarchitecture, and high-power insets demonstrate profiles in the retinal ganglion cell layer, inner nuclear layer, and outer nuclear layer (B, C, and D, respectively). The specific features used in cell classification are given in the Materials and Methods section entitled "Recognition of Cell Phenotype". B shows two neuronal profiles—the one on the right, labeled RGC, is of a size to be unequivocally identified as a ganglion cell, and that on the left, labeled RGC/dAm, is either a small ganglion cell or a medium-sized displaced amacrine cell. Abbreviations: same as in Figure 1, with, in addition: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; dAm, displaced amacrine cell. Scale bar = 10 μm in D (applies to B–D); 15 μm for A.
We used defined developmental “landmarks” that the retina passed through to specify cell genesis. Specifically, the ages at which 5, 50, and 95% of retinal cells became postmitotic were considered early, middle, and late stages of cell genesis, respectively (Fig. 3). Three stages of cell production can be recognized. An early, “ramp-up,” stage is first, in which the initial production of cells increases slowly over approximately 5 days (E10 –E15). This stage is well defined by the E10 –E15 data points that lie on a straight line ($r^2 = 0.98, P < 0.001$) with a slope indicating a rate of cell addition of 1.4 cells/hr/section (Fig. 3). After E15 this rate increases—the cumulative percent of cells rises linearly ($r^2 = 0.95; P < 0.001$, for data points E15–P6), until approximately P6 (Fig. 3). Fifty percent of all retinal cells had been generated between P1 and P2 and 95% by about P6. Cell genesis slows sharply for the production of the final 5% of cells. By P10 the density of $^3$H-TdR-labeled cells is very low, with a final, very small percentage of retinal cells generated over the next 3 days (P11–P13). The cessation of cell genesis is even slower than the ramping up; the initial 5% of cells is generated over 2 days, whereas the final 5% takes 5 days.

The central and peripheral zones of retina undergo comparable kinetics of cell production because the curves are similarly shaped (Fig. 3). However, there is a relatively consistent difference of 2–3 days in the timing of genesis of central and peripheral retina throughout all but the very first and last stages. Analysis of the time difference between central and peripheral retina demonstrates that the peripheral retina lags behind the center by, on average, 1.9 days ($\pm 11$ hours, SD).

**Spatial gradients of retinal cell genesis**

In the rat, as for other vertebrates studied, retinal cell birth starts in a central location and spreads to the periphery. The central and peripheral retina undergo comparable kinetics of cell production because the curves are similarly shaped (Fig. 3). However, there is a relatively consistent difference of 2–3 days in the timing of genesis of central and peripheral retina throughout all but the very first and last stages. Analysis of the time difference between central and peripheral retina demonstrates that the peripheral retina lags behind the center by, on average, 1.9 days ($\pm 11$ hours, SD).

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riphery. Such a progression is illustrated by histograms of the frequency of $^3$H-TdR-labeled cells in each of the 440-$\mu$m bins from the superior retinal margin, through the optic nerve head to the inferior margin (Fig. 4). Most heavily labeled cells (78%) produced by $^3$H-TdR injection at E10 were in the central half of the retina (solid black bars in Fig. 4, top histogram). At this stage the most peripheral bins were completely devoid of labeled cells. The production of postmitotic cells subsequently expanded into the peripheral bins—by E16 all bins contained some $^3$H-TdR labeled cells (data not shown). On the day of birth (P0), the proportion of $^3$H-TdR-labeled cells located in the central retina, although still a majority, was decreased to 61%. Five days later (P5), the balance shifted to the peripheral retina, which now contained 56% of heavily labeled cells. This resulted from both an increase in the density of cell genesis in peripheral retina and a local diminution in a small, central patch of five bins (2.2 mm), just superior to the optic nerve head (Fig. 4, P5–P32). The zone of diminished cell production rapidly expanded so that a four-bin (1.8-mm), central region was devoid of heavily labeled cells 1 day later (P6, data not shown), and by P9 (Fig. 4, bottom) the few labeled cells present were entirely confined to the three most peripheral bins (1.3 mm at each margin). Finally, at P13, the last age examined, only three labeled cells were observed in three sections, and all were at the furthest retinal margins (data not shown).

**Sequence of retinal cell genesis**

All cells heavily labeled with $^3$H-TdR from E10 injection were retinal ganglion cells, horizontal cells, or cones (Fig. 1). This conclusion is supported by observations of lightly labeled cells made from the E8 and E9 injected material. In this material, although no cell had a density of label exceeding the “heavy label” criterion, the highest grain counts were observed over retinal ganglion cell nuclei followed by horizontal cells and cones (data not shown).

The dynamics and sequence of production are illustrated in Figure 5, which plots the cumulative percent of cells generated at each age for each cell phenotype. To maximize resolution, the data plotted in Figure 5 are from the central sample. From this plot, we can determine the time that each cell type reaches our defined “landmarks” by dropping a vertical line from the intersection of each
cumulative plot with the 5, 50, and 95% lines to the abscissa (Fig. 5). These estimated ages are given in Table 1 for the central retina as well as the peripheral region and the retina as a whole.

Retinal ganglion cells were the first to reach the 5% level of genesis followed shortly after by horizontal cells and cones that arrived at very similar times (Table 1). The interval between the arrival of each cell type at each stage of development is given in Table 1 in the column labeled “Δ” for difference. These differences represent the estimated number of days and hours between when a developmental landmark is reached for one cell type minus the value for the succeeding cell type in the sequence. If there were no orderly sequence of cell genesis, these differences would as likely be positive as negative. However, for our proposed sequence of cell genesis, only 11 of 54 comparisons are in the negative direction. Such instances are indicated by a negative number, and italicized font. Most of these, “out of order,” pairwise comparisons are for the earliest cohort of the first and last generated cell types. For example, 5% of central cones were born 4.4 hours before the equivalent percent of horizontal cells in the same region. However, the full complement of data indicates that horizontal cells were generated before cones in rat, as they are in monkey (LaVail et al., 1991). We suspect, because the temporal resolution is poor (at least compared with the monkey), that this is an occasion when variability appears to switch the order. Retinal ganglion cell production, was overtaken by horizontal cells about one-third of the way through their genesis (Fig. 5). Thus, 50% of horizontal cells were generated 8.8 hours before an equal proportion of ganglion cells, and 40.4 hours before an equal proportion of cones (Table 1).

Ganglion cell production decelerates late, as is shown by the sudden decrease in the slope of this line after E15 (Fig. 5). As a result, the age at which 95% were produced remained behind horizontal cells by 4 days and was overtaken by cones, so as to precede them, at this point, by 1 day, 16.0 hours (Table 1). It is likely that the late deceleration in ganglion cell genesis results from the inclusion of displaced amacrine cells in these counts (see Discussion for further development of this argument). We have no independent means of distinguishing retinal ganglion cells from displaced amacrine cells; however, if the latter are generated later than ganglion cells, their inclusion in these cell counts would slow and lengthen the period of apparent ganglion cell genesis. The temporal relationships between the next two cell types, amacrine cells and rods, was consistent, with 5, 50, and 95% of the former generated substantially before corresponding proportions of the latter. Rod photoreceptor genesis significantly preceded a final cohort consisting of Müller and bipolar cells.
These two cell types appeared to become postmitotic at a similar time and at a very similar rate. For example, they reached the 50 and 95% cumulative percent levels at times differing by only 6.4 and 2.8 hours, respectively (Fig. 5, Table 1). Thus, Müller cells and bipolar cells appeared to be generated almost simultaneously.

**Size analysis of retinal ganglion cell layer genesis**

Some information about the cell types in the GCL can be garnered from their size because ganglion cells are generally larger than amacrine cells (Perry, 1981; Dreher et al., 1984; Reese and Colello, 1992). We measured the soma size of 3H-TdR-labeled and unlabeled neurons in the GCL from central and peripheral regions of en face sections. Heavily labeled cells were observed in the GCL of these sections from E10 to P2, confirming the data from radial sections. However, there were only sparse numbers at E10, and from E19 to P2, so the analysis was performed on E11–E18 tissue only. For each injection age the soma size of at least 100 cells was determined. Of these we determined which were heavily or lightly labeled with 3H-TdR. More profiles were measured in the tissue from relatively early or late injection so as to increase the sample of labeled cells. We pooled all the data (labeled and unlabeled cells, several thousand observations) to obtain a control population that accurately reflected the distribution of soma diameters of cells in the adult rat GCL (what we call the total population [TP]). The diameters of the TP in the peripheral retina ranged from 5.5 to 24.0 μm, with a mean of 10.4 μm (± 2.5 SD). This is shown in Figure 6B as a pair of thin horizontal lines delimiting the range of soma diameters (i.e., total population minimum and maximum) and a thick horizontal line indicating the mean. The cells heavily labeled by E11 injection (Fig. 6A) were between 8.2 and 13.1 μm diameter (mean = 10.9 μm ± 1.3 SD). This range is considerably constricted relative to the TP (Fig. 6B).

Between E11 and E17, the minimum soma diameter of labeled cells remained relatively constant, varying between 7.0 and 8.7 μm. During the same interval the maximum diameter of 3H-TdR-labeled cells increased, from 13.1 μm (E11) to 22.1 μm (E15), before decreasing from E15 to E18 (Fig. 6B). Between E17 and E18, late in the genesis of cells in the GCL, the profile of cells generated differed in their absolute number in the retina. Fewer of any phase 1 cell types were made than any phase 2 types. The relative proportion of each type can be estimated by measuring the area under their curves in Figure 7. Phase 1 cells constituted 10.6% of the cells when amacrine cells were excluded, or 18.8%, including amacrine cells. The significant difference with phase 2 reflects the number of rods generated; rods are the largest population (55%) of cells in the rat retina. Phases 1 and 2 also differ in their kinetics of cell production. Phase 1 cells begin genesis very rapidly, undergo a relatively constant rate of production for a prolonged run (E10—E16), and shut down their production relatively rapidly. These kinetics are shown by the steeply rising and falling slope, as well as the prolonged run, of each plot. These features give phase 1 cell curves a characteristic “plateau” shape (Fig. 7). The shape of the curves of the phase 2 cells is very different, now indicative of steadily increasing cell production to a maximum and a rapidly following, steady decrease. These curves can be described as “normal” or “bell-shaped” (Fig. 7) and describe an exponential function.

**Differences between early and late cell genesis**

The cumulative plots shown in Figure 5 illustrate that the production of cells did not proceed in smooth steps from one type to the next. For example, the distances between the 5, 50, and 95% landmarks are not equal between successive cell types. The first three cell types to be generated were somewhat clustered, so they reached developmental landmarks very close to each other. Subsequently there was a “pause,” apparent as a significant gap (Fig. 5), before the genesis of the next cell type, amacrine cells. A break of similar magnitude was seen between amacrine and bipolar cells. Finally, after a more moderate lag, the last cell types to be born, like the first, did so nearly simultaneously. The temporal “gaps” served to separate retinal cell genesis into two or three phases. The first, phase 1, included retinal ganglion cells, horizontal cells, and cones, and the last, phase 2, included rods, bipolar cells, and Müller cells. It is unclear whether amacrine cells should be considered a phase of their own or belonging to the first or last phase.

In addition to the temporal groupings, phase 1 and 2 cells differ in their absolute number in the retina. Fewer of any phase 1 cell types were made than any phase 2 types. The relative proportion of each type can be estimated by measuring the area under their curves in Figure 7. Phase 1 cells constituted 10.6% of the cells when amacrine cells were excluded, or 18.8%, including amacrine cells. The significant difference with phase 2 reflects the number of rods generated; rods are the largest population (55%) of cells in the rat retina. Phases 1 and 2 also differ in their kinetics of cell production. Phase 1 cells begin genesis very rapidly, undergo a relatively constant rate of production for a prolonged period (E10—E16), and shut down their production relatively rapidly. These kinetics are shown by the steeply rising and falling slope, as well as the prolonged run, of each plot. These features give phase 1 cell curves a characteristic “plateau” shape (Fig. 7). The shape of the curves of the phase 2 cells is very different, now indicative of steadily increasing cell production to a maximum and a rapidly following, steady decrease. These curves can be described as “normal” or “bell-shaped” (Fig. 7) and describe an exponential function.

Fig. 6. A: Histogram showing the soma size distribution of neurons in the ganglion cell layer of the P303 rat retina and the subpopulation heavily labeled with 3H-TdR injected at E11. The control sample ranges from 5 to 15 μm. In contrast, E11 labeled cells do not have any profiles at the smallest and largest ends of the size distribution. B: Graph illustrating the developmental trend of soma size for adult neurons in the retinal ganglion cell layer generated between E11 and E18. The mean, minimum, and maximum soma diameters are plotted, and error bars represent the standard deviation. For comparison, the same parameters of a large, control sample (total population) of neurons in the adult ganglion cell layer are represented by horizontal lines. The mean diameter of adult cells generated at E11 was not very different from the control, although the minimum and maximum are, as also seen in A, considerably different. The mean soma diameter of 3H-TdR-labeled cells gradually increases to 14.7. This reflects the generation of larger and larger cells (see trend in maximum), while the minimum soma diameter remains relatively constant. All parameters of ganglion cell layer neurons generated decrease from E15 to 17 and reach a minimum at E18. C: Histogram showing the soma size distribution of neurons in the ganglion cell layer of the P191 rat retina and the subpopulation heavily labeled with 3H-TdR injected at E18. The control sample ranges from 6 to 20 μm. In contrast, late generated cells are concentrated at the small end of the size distribution (6–11 μm).
Figure 6
Amacrine cells are a unique class sharing some features of phase 1 and phase 2. Although they exhibit a relatively steady, asymptotic level of genesis throughout most of the course of their production, similar to phase 1 cells, there was a slow ramp-up and ramp-down of their genesis much like phase 2 cells (Fig. 7). Finally, a close look at the curves for retinal ganglion cells and amacrine cells is instructive. The kinetics of the initiation of ganglion cell genesis closely resembled other phase 1 cells, rapidly reaching a plateau. However, the kinetics of cessation was long and drawn out, similar to the amacrine cell curve. Indeed, from E18 the ganglion cell curve departed from the other phase 1 cells and followed that for amacrine cells extraordinarily closely (Fig. 7).

A further difference was apparent in the spatial extent or topography of genesis of phase 1 and 2 cell types, as shown in the histograms of Figures 8 and 9, respectively. For each cell type three histograms illustrate the distribution of heavily labeled cells at time points close to the 5, 50, and 95% developmental landmarks. Early in development, postmitotic retinal ganglion cells, horizontal cells, and cones were found nearly throughout the extent of the retina except the most peripheral two or three bins at the superior and inferior margins (Fig. 8). The later generated cell types extended less into the periphery when only about 5% had been born (Fig. 9). In most instances at least four or five empty bins were present at each margin. At late stages of development the density of $^3$H-TdR-labeled cells was sparse for ganglion cells, horizontal cells and cones, and they were distributed in bins spanning the extent of the retina (Fig. 8). In contrast, Figure 9 illustrates a clear and extensive area of the central retina where genesis of phase 2 cells has ceased entirely whereas it remained ongoing in the periphery. In topographic patterning of cell genesis amacrine cells resemble phase 2 cell types.

**DISCUSSION**

**Initiation of the neurogenetic period in the rat retina**

With the advent of the cell “birthdate” technique using pulse labeling with $^3$H-TdR, one of the first tissues studied...
was the mouse retina (Sidman, 1961). Since this pioneering work several similar studies have been published, but they have been limited in a number of ways. Some have focused solely on postnatal cell genesis (Blanks and Bok, 1977; Young, 1985a), whereas others have studied only certain cell layers or cell types (Carter-Dawson and La-Vail, 1979b; Dräger, 1985; Evans and Battelle, 1987; Zhang and Yeh, 1990; Reese and Colello, 1992; Galli-Resta and Ensini, 1996; Ilia and Jeffery, 1996; Lee et al., 1999; Ilia and Jeffery, 2000; Rachel et al., 2002). Many have not been able to recognize different cell types (Sidman, 1961; Gloor et al., 1985; Ilia and Jeffery, 2000). Finally, most have not traced retinal cell genesis back to the stage where it has yet to begin or to when it is entirely finished. This study was undertaken to provide a complete description of the genesis of all retinal cells types from beginning to end, in the laboratory rodent.

No cells in retinas exposed to ³H-TdR on E8 or E9 were heavily labeled in the mature retina, but a small number were seen following injection on E10. Thus, we conclude that cell genesis in the rat starts between E9 and E10. This corresponds, approximately, to the 4–13-somite, 2-mm crown-rump, stage, when the retina is an optic vesicle (Hebel and Stromberg, 1986). The only prior rat study to trace genesis back to the zero point focused on retinal ganglion cell genesis, the first cells born. Tritiated thymidine injections on E12 or E13 failed to produce labeled ganglion cells from E12 injection (Galli-Resta and Ensini, 1996; Ilia and Jeffery, 1996) but did not look earlier. Given the slow ramp-up of retinal cell genesis over the first 4 or 5 days (Fig. 3), it is possible that extrapolation of these data could demonstrate an intersection with zero “percent

Fig. 8. Histograms showing the topography of generation of early born, phase 1, cell types in the rat retina. The number of cells from three sections is shown, with each bin representing a 440-μm linear segment of retina from the superior to the inferior margins, at the level of the optic nerve head (ONH). For each cell type, data close to the early (5% of cells generated), middle (50% of cells generated), and late (95% of cells generated) stages of retinogenesis are shown. Labeled cells are found over most of the retina throughout development, with a tendency not to extend into the most peripheral bins early and to be less dense in the central bins late.
Figure 9
cells labeled” around E10. In the face of this discrepancy with earlier work, it is only safe to say that retinal cell genesis in the rat begins between E10 and E13. Indeed, the technique used to determine this range, pulse labeling, is unlikely to offer greater precision. This method labels the cohort of cells that stop dividing within a short interval after administration of a DNA synthesis marker with a very short biological half-life. Technical considerations such as the rate of uptake of the label, length of the label’s availability, time to availability to the developing eye, and other factors make detecting the small cohort of labeled cells present at the initiation of cell genesis particularly difficult to resolve. On the other hand, these confounding factors can be controlled for the significant number of labeled cells present throughout most retino genesis.

A more suitable way to determine the initiation of cell genesis is through cumulative labeling. A label of DNA synthesis is administered at a defined stage and continuously replenished throughout the genesis of the structure under study. When analyzed, all cells that were dividing after the injections began are labeled, and the only unlabeled profiles are those that were born earlier. These are obvious against the dense background of heavily labeled cells, and there is no need to define arbitrarily what constitutes a labeled cell. Cumulative labeling was initially applied, by necessity, to closed developmental systems like bird eggs (Kahn, 1974) or bowls of fish water (Hu and Easter, 1999; Otteson et al., 2001). In these environments a pulse cannot be administered because there is no route for the label to escape or dilute. However, in mammals, the short biological half-life of 3H-TdR and bromodeoxyuridine (BrdU) is well established (Denham, 1967; Skougaard and Steward, 1967; Blen kninsopp, 1968; Nowakowski and Rakic, 1974; Hickey et al., 1983; Young, 1985b; Alexiades and Cepko, 1996). Therefore, to achieve cumulative labeling many injections must be made throughout development; if one is seeking the first cells to be born, this can involve considerable expense, time, and effort (Nowakowski et al., 1989; Takahashi et al., 1993). For example, following protocols established for the mouse cortex, cumulative labeling of rat retina from E10 to the cessation of ganglion of cell genesis would involve injections of 3H-TdR or BrdU to pregnant dams up to eight times a day, over 10 days. Until this experiment is performed, the time of initiation of rat retinal cell genesis and, correspondingly, retinal ganglion cell birth must remain undefined between E10 and E13.

Timing of genesis of specific retinal cell types in the rat

Because ganglion cells are the first cells in the retina to be born, the preceding discussion of the timing of the initiation of cell genesis in the retina applies to ganglion cells. Amacrine cells genesis has been studied in the rat previously (Evans and Battelle, 1987; Zhang and Yeh, 1990; Reese and Colello, 1992; Lee et al., 1999). Investigators have been primarily concerned with specific subsets of amacrine cells determined by immunoreactivity to different neurotransmitters or their synthetic enzymes. Taken together, they define a broad window of amacrine cell genesis, beginning before E13 and ending in the postnatal period. Within this window, different subtypes have different periods of genesis. γ-aminobutyric acid (GABA)ergic cells are heavily labeled with a birthday marker injected prior to E13 (Lee et al., 1999), whereas the comparable experiment for cholinergic, dopaminergic, or corticotropin-releasing factor (CRF)ergic amacrine does not produce heavily labeled cells until E15–16 (Evans and Battelle, 1987; Zhang and Yeh, 1990; Reese and Colello, 1992). We see postmitotic cells lining the vitreal margin of the INL of the central retina after injection at E10, but they are quite rare until injected at E13.

Furthermore, there is a possibility that these are not amacrine cells but displaced ganglion cells (cells with somata at the innermost margin of the INL, extending axons into the optic nerve). In the mouse (Dräger, 1985) and rat (Liu and Jen, 1986; Landen, 1987; Buhl and Dann, 1988), these form approximately 1–2% of the ganglion cell population. If ganglion cell genesis starts at E10, some small proportion would be located in the INL because the birthdates of displaced and normally placed ganglion cells entirely overlap (Dräger, 1985). If the earliest labeled cells at the inner margin of the INL are ganglion cells, our data agree with prior studies timing amacrine cell birth. Some of the difference between our data on rat amacrine cell genesis and others may result from eccentricity differences, a factor that was not accounted for in many of the prior studies. It may also be significant that the late generated amacrine cell subtypes are a relatively small fraction of the total population of amacrine cells. It has been estimated that 40% or more of amacrine cells are GABAergic, and a correspondingly large population are glycinergic (Vaney, 1990). Thus, the birth of GABA-containing amacrine cells would seem to be most representative of this cell class as a whole, and the birthdates of these (Lee et al., 1999) are close to our data. Photoreceptor genesis has been reported for the rat (Ilia and Jeffery, 2000). Although these investigators did not trace cell genesis to the beginning, and did not distinguish between rods and cones, their data support our timing. They note a small cohort of cells in the ONL that were heavily labeled by 3H-TdR injected at E12. Our data indicate that, of the cells in the ONL, only cones are being generated at this early stage. Ilia and Jeffery did not look any earlier than E12, but, given the small number of cones in the rat, it is likely that what they report represents a significant proportion of the total cone population that was generated by E12. Rod genesis peaks between the day of birth and P2 and terminates after P6, data that agree with the findings of Ilia and Jeffery (Ilia and Jeffery, 2000).
Rat retinal cells are produced in a defined order

Different retinal cell types are generated simultaneously throughout most rat retinogenesis. Between E15 and E18, all types are being generated (Fig. 7). However, at this stage, some are near the end of their proliferative period whereas others are beginning. This is why the cumulative graphs (Fig. 5) and developmental landmarks (5, 50, and 95% of cells generated) are so helpful in ultimately specifying the sequence of genesis. At most of the developmental landmarks, retinal cell genesis can be seen to occur in the following sequence (from early to late): retinal ganglion cells, horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and Müller cells. This order, except for some slight differences (to be discussed), is the same as that seen in monkey (LaVail et al., 1991) and quokka wallaby (Harman and Beazley, 1989), the only other mammals in which a complete analysis of retinal cell genesis order has been performed. These data are also in substantial agreement with other partial studies that suggest aspects of a retinal cell genesis sequence in a variety of mammals (mouse, Sidman, 1961; Blanks and Bok, 1977; Carter-Dawson and LaVail, 1979b; Dräger, 1985; Gloor et al., 1985; Young, 1985a; Evans and Battelle, 1987; rat, Zhang and Yeh, 1990; Reese and Colello, 1992; hamster/gerbil, (Sengelaub et al., 1986; Wikler et al., 1989; cat, Walsh and Polley, 1985; Zimmerman et al., 1988) and non-mammals (chick, Kahn, 1974; Prada et al., 1991; goldfish, Sharma and Ungar, 1980; zebrafish, Hu and Easter, 1999).

The lines plotting cumulative percent of cells generated hardly ever cross (Fig. 5), indicating that the ordinal position of each cell type remains relatively constant. The single departure from this observation is among the first cells generated. Retinal ganglion cells reach the 5% landmark before any other cell type but are surpassed by horizontal cells at about 30% of cells generated (Fig. 5), and then by cones at about 90% of cells generated. These data could suggest a variable ordinal relationship among these three cell types. However, we would argue that this is rather a reflection of the fact that the GCL contains two distinct types of neurons that have different periods of genesis. Specifically, the GCL contains retinal ganglion cells and amacrine cells (usually referred to as displaced amacrine cells). The population of the later, in the rodent, is substantial, constituting 50–60% of the neurons in the GCL (Perry, 1981; Jeon et al., 1998). A number of features of the genesis of neurons in the GCL indicate that these two neuronal types are born at different times. One feature is the length of the proliferative period of the cells in this layer, which is longer than that of most other cell types, particularly the early (phase 1) ones. This results almost entirely from a very gradual decrease in cell production (Fig. 5, retinal ganglion cell plot) that could reflect the inclusion of a later generated cell type. A similar feature was observed in the ganglion cell layer of monkey retina, although it was less pronounced (see Fig. 18 in LaVail et al., 1991). Analysis of the size of neurons in the GCL, although far from definitive, can be helpful in identifying the two cell types contained. The diameter range of neurons in the GCL of the rat is 5–22 μm (Fukuda, 1977; Perry, 1981; Dreher et al., 1985), and our data (Fig. 6) are in accord. Of these, amacrine cells are smaller, ranging from 5 to 14 μm in diameter (Perry, 1981; Mitrofanis et al., 1988; Mitrofanis, 1989; Kim et al., 2000; Oh et al., 2002). Retinal ganglion cell diameters overlap with amacrine cells at the small end of the distribution, but can be much larger, 8–22 μm (Perry, 1981; Huxlin and Goodchild, 1997).

With this in mind, we can interpret the trends in the size of GCL neurons born between E11 and E18 (Fig. 6). The earliest of these (E11) have some diameters that include large amacrine cells and small to medium retinal ganglion cells (Fig. 6A). We hypothesize that these are primarily ganglion cells, because the diameter range does not include the “exclusively amacrine” portion of the size spectrum, whereas it does contain cells that are unequivocally ganglion cells based on size. With development, the soma diameter range expands to include increasingly larger cells, including many more beyond the amacrine cell size range (Fig. 6B). The same pattern was seen in cat and primate retinas (Walsh and Polley, 1985; Rapaport et al., 1992). At late stages the size distribution suddenly constricts and shifts to lesser values, now including the entire range of diameters of amacrine cells, but only small to medium ganglion cells (Fig. 6C). We suggest that the late generated cells are displaced amacrine cells. This interpretation is consistent with previous studies showing that displaced amacrine cells in the rat begin their genesis after ganglion cells (Reese and Colello, 1992). In this light, it is not surprising that the cumulative frequency curve of percent of ganglion cells generated (Fig. 5) is crossed by horizontal cells and cones and flattens out at late stages. This is the inevitable result of including an increasing number of displaced amacrine cells in the dataset.

One of the most difficult sequential relationships to resolve is between the last cell types to be generated. Initially, Müller cells precede bipolar cells. The relationship switches near the middle of their genesis and again just prior to their termination (Fig. 5, Table 1). The frequent reversals could be a reflection of nearly simultaneous genesis of these two cell types. In the monkey, although Müller cells consistently preceded bipolar cells, the interval was the smallest of any sequential pair, only 3–5 days (LaVail et al., 1991). The specific identity of the last cells generated is of interest because of a recently proposed model of gliogenesis suggesting that cell cycle termination and glial fate determination are intimately linked (Dyer and Cepko, 2000; Vetter and Moore, 2001). Although not a requirement of this model, it would fit well if the fate expressed at terminal cell division were a Müller cell one. The data presented here suggest this is a strong possibility. However, refined techniques will be needed to address the question of whether Müller and bipolar cells are generated contemporaneously with variable ordinal relations, or whether one precedes the other in an as yet irresolvable sequence.

Although variability in the ordinal relations between cell types was rare, it was more frequent in the rat than in the monkey (compare Fig. 5 with Figs. 18 and 19 in LaVail et al., 1991). This probably reflects the rapid development of the rat. In the monkey most retinogenesis occurs over 100 days, whereas the comparable period for the rat is 21 days. Having to make the same cell types over a shorter interval degrades the ability to resolve small differences. The critical question is whether the poor resolution reflects an inability of retinal progenitor cells (RPCs) to follow a developmental sequence, or simply a technical inability to resolve the temporal aspect adequately. The
former would suggest that at any stage of retinogenesis there is a bias to make certain cell types but that cell fate determination also has a stochastic component. Thus, there would be a low probability that progenitors would generate cells widely separated on the timeline, say horizontal and Müller cells, in a reversed order. However, cell types close together in the sequence, such as ganglion cells, horizontal cells, and cones, or bipolar and Müller cells, might have a nearly equal probability of being generated one before the other or vice versa. Alternatively, there could be many retinal progenitors producing postmitotic daughters at the same time. If each were at a slightly different stage of cell production, even if the order that they produce cells is rigid, construction of the interval of cell genesis would increase the apparent variability in the sequence of cell production when the population is considered. To distinguish between these two hypotheses, one needs to examine cell birth order within the progeny of single RPCs.

The only significant departure in the sequence of cell genesis in the rat retina compared with the monkey and wallaby is the relatively early position of rods. In rat, rods are generated as an early component of the “late” phase of cell genesis. In comparison, in the monkey, rods appear to be one of the last cell types generated (LaVail et al., 1991). The rat retina is rod dominated; they constitute approximately 97–99% of photoreceptors (LaVail, 1976; Carter-Dawson and LaVail, 1979a; Szel et al., 1996; Jeon et al., 1998). In the primate, although rods significantly outnumber cones, cones are the exclusive photoreceptor at the center of the fovea, predominate up to 0.5 mm eccentricity, and are the majority photoreceptor until about 3 mm from the fovea (Packer et al., 1989; Wikler et al., 1990). The monkey data were collected from sections through the fovea and therefore over-represented cones as a proportion of the total population of photoreceptors. However, when the sampling area was confined to the periphery, the position of rods in the order of cell genesis shifted from last (total and central samples) to fifth (see Fig. 19B in LaVail et al., 1991). In this situation the order is the same for the rat and the monkey. These data suggest that cell genesis can be influenced by the absolute number of cells to be generated—the more cells to be made, the earlier genesis begins.

**Early and late phases of retinal cell genesis**

Besides the approximate sequence, another feature of retinal cell genesis that appears to be highly conserved is the presence of distinct phases of cell birth. Here we distinguish two, phases 1 and 2, which differ in timing, kinetics, and topography of production. Amacrines cells share some properties with each phase and may constitute one of their own. Two phases of cell genesis have been seen in the wallaby (Harman and Beazley, 1989) and monkey (LaVail et al., 1991) retina. In the wallaby, they are separated by approximately 20 days of mitotic quiescence, whereas in the monkey, and now the rat, they are overlapping. Phase 1 genesis is rapid and regular and exhibits the least dramatic central-peripheral gradients. The cell types constituting phase 1, ganglion cells, horizontal cells, and cones, have strong central-peripheral gradients of distribution in the adult (i.e., high in the center, low in the periphery), suggesting a relationship between these developmental and adult features (Harman and Beazley, 1989; LaVail et al., 1991). Specifically, rapid and evenly distributed cell genesis provides a uniform template of early cell phenotypes from which an adult distribution is shaped by mechanisms such as differential retinal expansion, apoptosis, etc. For phase 2 cells, the effects of these differential mechanisms are at least partly counteracted by differential cell production (as shown by strong central-peripheral gradients of 3H-TdR-labeled cells). These cell types therefore exhibit a more regular distribution across the surface of the retina. Another model suggests that early cells are generated more uniformly across the retina to serve as a template for the determination and differentiation of subsequent cells. For example, an evenly distributed array of early differentiating cones forms a “proto-map” of their mosaic in the monkey retina (Wikler and Rakic, 1991), and a similar mechanism may be operating for phase 1 cells in the rat.

Phase 1 and 2 cells also differ considerably in their numbers. Specifically, phase 1 cells constitute a significantly smaller proportion of the cells in the retina than those of phase 2. Reflecting this, they show strikingly different kinetics of production. It is widely thought that neural progenitors undergo a number of symmetrical divisions until they reach a density to make the appropriate number of postmitotic cells in the available time. At this point it is suggested that they switch to asymmetric mode—producing a postmitotic daughter and returning a mitotic progenitor. Phase 1 cells appear to exhibit classical steady-state kinetics and fit this model well. However, phase 2 demonstrates an exponential increase in cell production. We hypothesize that progenitors replenish themselves by undergoing one or more symmetrical divisions between phase 1 and 2. Such a mechanism accommodates the proliferative demand for these cell types. The consistently observed “pause” in cell genesis between phases would be expected were progenitors going through a period of symmetric cell divisions. Zebrafish retina is an extreme example of such a case; all retinal ganglion cells are made by an early wave of neurogenesis, whereupon the cell cycle proceeds with symmetric divisions for 10 hours and no cells become postmitotic (Hu and Easter, 1999; Li et al., 2000). A similar, although much more drawn out, example may be the 20 days separating phases 1 and 2 in the wallaby (Harman and Beazley, 1989). Variation in the length of the “between-phase” period might reflect the relative proportion that phase 2 cells constitute in the retina of different species.

**Extrapolating the rat data to the mouse**

With recent knowledge of the murine genome, and the technology to alter gene function, more and more research is being conducted on the rat’s rodent cousin, the mouse. Although there is a literature on mouse retinal cell genesis (Sidman, 1961; Blanks and Bok, 1977; Carter-Dawson and LaVail, 1979b; Young, 1985a), no complete study has been performed. The Young (1985a) paper that is most often cited is based on postnatal injections of 3H-TdR, even though most cell genesis takes place before birth (Sidman, 1961). In addition, it appears as though the summary that Young presents of his and Sidman’s data has been significantly smoothed because the cell genesis graphs he shows are symmetrical. We see normal curves only for the late generated cell types (Fig. 7), and they are not smooth, reflecting variation between animals injected at different ages.
Because retinal development is similar in many vertebrates (for review, see Robinson, 1991), it is suggested that a common timetable can be scaled to fit any species. One scaling factor is the time from conception to eye opening, the caecal period (Robinson and Dreher, 1990). Robinson and Dreher show that many aspects of visual system development, including cell genesis, occur at common time points relative to the caecal period. We feel it would be useful to scale the timetable of rat retinal genesis to generate a hypothetical one for the mouse (Table 2). We hope that these data can provide a guide to those interested in retinal development of the mouse until the empirical studies are done.

The rat caecal period is 36 days (P14), and that of the mouse is 29 days (P10) (Green, 1966; Hebel and Stromberg, 1986). Therefore, a scaling factor of 0.806 (caecal period mouse/caecal period rat), applied to our rat cell birth data predicts the time of genesis of retinal cell types in the mouse. These data are presented in Table 2, which recapitulates the developmental landmark data of Table 1 for the whole retina. For example, we suggest that 5% of mouse retinal ganglion cells are born between E9 and E10.

<table>
<thead>
<tr>
<th>Cell</th>
<th>5%</th>
<th>50%</th>
<th>95%</th>
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<tr>
<td>RGC</td>
<td>E1 + 2.4 h</td>
<td>E11 + 0.8 h</td>
<td>E16 + 0 h</td>
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<tr>
<td>Ho</td>
<td>E8 + 0.4 h</td>
<td>E10 + 0.8 h</td>
<td>E12 + 18.5 h</td>
</tr>
<tr>
<td>CPv</td>
<td>E7 + 12.9 h</td>
<td>E12 + 3.4 h</td>
<td>E14 + 14.9 h</td>
</tr>
<tr>
<td>Am</td>
<td>E10 + 6.9 h</td>
<td>E14 + 14.9 h</td>
<td>P0 + 7.0 h</td>
</tr>
<tr>
<td>RPr</td>
<td>E14 + 17.4 h</td>
<td>P0 + 8.0 h</td>
<td>P3 + 8.1 h</td>
</tr>
<tr>
<td>BP</td>
<td>E17 + 16.4 h</td>
<td>P1 + 15.5 h</td>
<td>P4 + 3.8 h</td>
</tr>
<tr>
<td>Mu</td>
<td>E17 + 3.2 h</td>
<td>P2 + 2.1 h</td>
<td>P4 + 8.2 h</td>
</tr>
</tbody>
</table>

RGC, retinal ganglion cell; Ho, horizontal cell; CPv, cone photoreceptor; Am, amacrine cell; RPr, rod photoreceptor; BP, bipolar cell; Mu, Müller glial cell.

Models of vertebrate retinal cell fate acquisition (Perron and Harris, 2000; Livesey and Cepko, 2001; Marquardt and Gruss, 2002) suggest that two mechanisms act in concert. Molecular signals in the environment activate transcription factors that move uncommitted progenitors to a progressively more differentiated state. The identity of these inducers is being actively pursued, and several promising candidates have been proposed (Harris, 1997; Cepko, 1999). Because much of the work attempting to identify inducers has been performed in the rat, knowledge of the time of genesis of different retinal cell types is important in narrowing the search for operational molecules and when they exert their affect. In addition, RPC’s must be in a state to respond to these inducers. This property of competence is cell autonomous and may run on an endogenous clock, so that progenitor cells become successively able to respond to cues to become a ganglion cell, then a horizontal cell, then a cone, etc. Were this the case, studies of retinal cell genesis will define the parameters of when, where, and in what order cells become competent to adopt a particular fate.

In addition to making the right types of cells during development, the right number of each has to be made. We suggest that ordered cell birth contributes not just to fate acquisition but also to making the correct quantity. Indeed, we see different kinetics for genesis of early and late born cell types and corresponding differences in their overall contribution to the retina and their topographic distribution. In this context, it is interesting that rods are generated relatively early in rat compared with monkey perifovea, but in the same ordinal position when the peripheral retina is considered. Within the highly conserved order of retinal cell genesis, variation appears to reflect the prominence of a cell type in the retinal population.

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LITERATURE CITED


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