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APPENDIX 1

Histological Procedures for Normative Embryonic Studies

Wistar female rats were transferred to the home cages of individually housed Wistar male rats from 4:00 P.M. to 7:00 A.M. the next morning. Vaginal smears were taken from each female, and those that contained sperm were placed into maternity cages. The date when sperm were found in the vagina was considered to be the first day of gestation, or embryonic, day (E) 1. Food and water was provided ad lib throughout gestation. Between 8:30 and 9:00 A.M. on each day from E13 to E22, two or more dams were deeply anesthetized with sodium pentobarbital (35 mg/kg) and the embryos were removed and killed by immersion in Bouin's fixative; from E17 on, the embryos were decapitated before fixation, and from E19 on, the calvarium was removed after the heads had been fixed for approximately 20 minutes to aid penetration of the fixative into the brain. The embryos remained in Bouin's fixative for 24 hours, then were transferred to 10% neutral Formalin (pH 7.4) until the time of embedding either in paraffin (Tissue Prep, Fisher) or in methacrylate (JB-4, Polysciences) according to standard histological procedures. Only well preserved specimens were cut serially at 10 μm (paraffin, every section saved) or 3 μm (methacrylate, every tenth section saved) in the three cardinal planes and were stained with hematoxylin (paraffin) or toluidine blue (methacrylate).

APPENDIX 2

Tritiated Thymidine Autoradiographic Methods

2.1 LONG-SURVIVAL AUTORADIOGRAPHY

2.1.1 Critique of the Pulse-Labeling Method

The possibility of experimentally dating neuronal "birthdays" began with the application of [^{3}H] thymidine autoradiography to neuroembryological research (Angevine and Sidman, 1961; Miale and Sidman, 1961). For about 15 years, the technique most commonly used with mammals was the pulse-labeling procedure, and some laboratories continue to use that method today. A single injection of [^{3}H]thymidine is administered (usually intraperitoneally) to a pregnant dam at a known gestational age. The offspring of these dams are allowed to survive into the juvenile and adult periods when the central nervous system is partly or fully mature. The question asked is: "Which group of cells and what proportion of these become heavily labeled?"

The heavily labeled cells are assumed to originate on the same day that the injection is given. However, the validity of that assumption cannot be experimentally tested because no one knows how soon after incorporation of label that a neuronal precursor actually produces a nonmitotic neuron. In our experience, it is common to see heavily labeled cells one or two days before the onset of neurogenesis. Thus, the assumption that all heavily labeled cells originate within 24 hours after the injection is not true for at least some of them, and pulse labeling studies will often erroneously date neuronal birthdays to occur earlier than they actually do.

In addition, there are problems in both defining and determining what constitutes a heavily labeled cell. That is due to the circumstance that label concentration over nuclei containing radioactive DNA depends on a host of factors other than imminent cessation of cell division. (1) Mitotic cells at the end of S-phase will incorporate less label than those in S-phase during the entire time of [^{3}H]thymidine exposure. Therefore, in spite of its light labeling, a cell might originate soon after the injection. (2) Cells with large nuclei often never become heavily labeled while small cells may be judged to be heavily labeled even after repeated divisions. (3) The extent of label concentration is also affected by the dose and specific activity of the radiochemical, by the sensitivity of the photographic emulsion, the duration of photographic exposure, and several other factors.

The pulse labeling technique concentrates on intensity of label as the source of data because one problem arises regarding the ambivalent birthdays of the unlabeled cells. Some neurons may be unlabeled because they are already postmitotic when the injection is given, while others are unlabeled because repeated cell divisions of their precursors dilute the incorporated label beyond the detection level. But an analysis of only the heavily labeled cells does not allow for a quantitative determination of the exact temporal order of neuron production in a specific neuronal population. The second problem with pulse labeling is that only a small proportion of the cells in a specific neuronal population tend to be heavily labeled (by any definition) after a single [^{3}H]thymidine injection, and therefore most of the cells cannot be included in data collection. Although some of these ambiguities can be resolved by serial injections of different groups of animals at different embryonic ages, the pulse-labeling method has never generated reliable quantitative data in long-survival studies. A modified long-survival [^{3}H]thymidine autoradiographic procedure is now available that allows the exact quantification of the proportion of neurons generated on specific days throughout the rat nervous system.

2.1.2 Cumulative Labeling Method

2.1.2.1 Experimental Design

The cumulative method uses multiple injections of [^{3}H]thymidine instead of single injections and makes a distinction only between labeled and unlabeled cells. This circumvents the label intensity and label dilution problems in the pulse-labeling technique and removes the ambivalence in determining the time of origin of the unlabeled cells. With this technique, therefore, the entire cell population is used as a source of data. The procedure consists of three main steps: (1) Groups of pregnant rats or postnatal animals are injected subcutaneously with two to four successive daily doses of [^{3}H]thymidine (Schwarz Mann, specific activity 6 Ci/mM, 5 μCi/gram body weight). As long as the injections in a single animal are separated by 24 hours, there is no radiation-induced cell death; brains of the injected animals appear normal in both size and cell number. Intraperitoneal or intravenous injections result in rapid removal of labeled precursor; 1 hour after the injection, only 5–10% of the labeled thymidine is still present in the blood (Cleaver, 1967). Feinendegen (1967) states that [^{3}H]thymidine is available longer after subcutaneous injections, but no specific length of time is given. The subcutaneous method of injection does not give a pulse label of [^{3}H]thymidine and is better suited for cumulative labeling. (2) The onset of the injections is progressively delayed between groups. For animals in the prenatal groups, injections on specific embryonic (E) days are set up in an overlapping series with 24 hour delays between groups (E12 + E13, E13 + E14 . . . E21 + E22). For animals in the postnatal (P) groups, the injection groups are separated by 48 hour delays, each group receiving either four (P0 − P3, P2 − P4 . . . P6 − P9) or two (P8 − P9, P10 − P11 . . . P20 − P21) consecutive daily injections. These injection schedules give a complete picture of neurogenesis throughout most structures in the rat central nervous system. (3) The animals are killed as adults (P60) so that the patterns of labeled and unlabeled cells can be examined in a fully mature brain.

2.1.2.2 Histological and Autoradiographic Procedures

All animals are deeply anesthetized with pentobarbital and are killed by perfusion with 10% neutral formalin through the heart. The brains are stored in Bouin's fixative for 24 hours and are then transferred to 10% neutral formalin until further processing. To compile the data for this book, the brains of at least six males from each injection group were blocked in the coronal plane, embedded in paraffin, and were sectioned serially at 6 μm (every 15th section is saved). Four to six brains from each injection group were blocked with a midline sagittal cut before paraffin embedding. One-half was serially sectioned horizontally, the other half coronally (both planes: every 15th 6 μm section is saved). The sections were mounted on slides with albumin adhesive. The afternoon before autoradiography, the sections were defatted in xylene and were rehydrated in graded alcohols to water. The slides were placed into a dust-free area in the darkroom overnight and were not allowed to become "bone" dry. All procedures in the darkroom were done using safelights fitted with series-2 filters (dark red). Beginning the next morning, the surface of each slide was coated with a thin layer of liquid photographic emulsion (Kodak NTB-3) warmed to 40°C. The slides were dried upright in metal trays for approximately 1 hour in a humid atmosphere (80–85%) and were loaded into metal staining trays. The trays were stored with a dessicant in light-tight metal boxes in the refrigerator for a 12-week exposure period. The autoradiograms were developed in Kodak D-19 and were poststained with hematoxylin and eosin.

2.1.2.3 Quantitative Evaluation

The cumulative labeling procedure does not require a distinction between heavily and lightly labeled cells. All neurons within a designated area are counted and are assigned to one of two groups; labeled or nonlabeled.[1] Cells with reduced silver grains overlying the nucleus in densities above background levels are considered labeled; obvious endothelial and glial cells are excluded. The proportion (%) of labeled cells are then calculated from these data, Eq. (1):

$$[\#\text{ labeled cells} / \#\text{ total cells}] \times [100] = \%\text{ labeled cells} \quad (1)$$

Our previous work has established that a chronological schedule of two successive [^{3}H]thymidine injections can be found that will label between 95% and 100% of the neurons in most rat brain structures. Table 1 gives a typical example of the data for neurogenesis of the Cajal-Retzius cells (Chapter 3, Fig. 3–2A). At the time of the maximal labeling level (E13-E14), all the precursor cells of these neurons are still proliferating (100% of the neurons are labeled), and none of the neurons have originated. By progressively delaying the onset of the injections in 24 hour intervals, the

[1] The cell counts were done using a Compac computer (DeskPro). Two of the function keys were programmed to represent labeled and unlabeled cells, and the raw data was sorted and analyzed in a series of related programs written by James Simmons, formerly an undergraduate student in Computer Science at Purdue University in West Lafayette, IN. Mr. Simmons is currently employed at Northrop Corporation.

TABLE 1. *Neurogenesis of the Cajal-Retzius cells*[a]

Injection group	N	% Labeled cells (Mean ± S.D.)[b]	Day of origin	% Cells originating[c]
E13–E14	8	(A) 100 ± 0	E13	5.61 (A – B)
E14–E15	7	(B) 94.39 ± 3.23	E14	63.50 (B – C)
E15–E16	7	(C) 30.89 ± 5.77	E15	14.72 (C – D)
E16–E17	7	(D) 16.16 ± 8.57	E16	11.67 (D – E)
E17–E18	7	(E) 4.50 ± 1.45	E17	4.40 (E – F)
E18–E19	6	(F) 0 ± 0	E18	0

[a] The data for the Cajal-Retzius cells are given as an example of how they are derived for presentation in the bar graphs used throughout the book on time of origin determined by long-survival [^{3}H]thymidine autoradiography (Chapters 3 and 11–15). *N* refers to the number of animals analyzed in each injection group. The "% Labeled cells" for each injection group gives the group means ± the standard deviation for the raw data counts (% of labeled cells to total cells in individual animals). The standard deviations are typical of the variability seen throughout data collection.

[b] S.D., standard deviation

[c] Graphed in Fig 3–2A, top.

percentage of labeled neurons declines. This reflects the production of postmitotic neurons by their precursor cells. The proportion of neurons originating each day is equal to the daily decline in the percentage of labeled neurons. For example: (94.39% labeled cells on E14 and E15) − (30.89% labeled cells on E15 and E16) = 63.5% cells formed during E14 (or between the onset of the injections on E14 and E15). Therefore, with this procedure the onset of generation can be exactly determined, and the proportion of cells produced each day can be calculated. These data allow for statistically evaluated comparisons between formation times of various neuronal populations and also for the detection of subtle neurogenetic gradients within structural subdivisions.

2.2 SHORT- AND SEQUENTIAL-SURVIVAL AUTORADIOGRAPHY

The pulse-labeling method, fraught with technical problems in long-survival studies, is ideally suited for short-survival autoradiography where the period of analysis is usually restricted to the embryonic and early postnatal periods. Within 2 hours after a single injection of [^{3}H]thymidine for example, the neuroepithelium and the secondary germinal matrices derived from it reveal themselves as heterogeneous patches containing regions of high or low label uptake. The pattern changes depending on the time of the injection. When the survival time is increased to daily intervals after the injection, heavily labeled migrating neurons can be followed from their germinal sources to their final locations.

The morphogenetic studies discussed in this book were based on observations of an extensive library of histological material from over 1,200 embryos and pups. Several pregnant female rats for each embryonic day from E13 to E22 were injected with a single dose of [^{3}H]thymidine (Schwarz-Mann; specific activity 6.0 Ci/mM; 5 μCi/g body weight). Survival times for each injection group varied from 2 hours (short-survival) to daily intervals for several days (sequential-survival). For example, one dam in the E15 injection group was killed 2 hours after the injection, another was killed 1 day later on E16, another 2 days later on E17, and so on, until the last dam was killed on E22. All groups were treated as the E15 group. In order to get a more complete developmental series for the late-prenatal originating neurons, injection groups from E19 to E22 included some survival times extending to P5. At the time of sacrifice, the dams were deeply anesthetized with sodium pentobarbital, and their embryos were removed and killed by immersion in Bouin's fixative. After further storage in 10% neutral formalin, both the head and body of each embryo was embedded either in paraffin or in methacrylate. The blocks were serially sectioned (at 6 μm in paraffin embedded material; 3 μm for methacrylate) in either the sagittal or coronal planes. Postnatal animals were killed by transcardiac perfusion with 10% neutral formalin. The brains were stored in Bouin's fixative for 24 hours, then were transferred to 10% neutral formalin until the time of embedding. The histological and autoradiographic procedures follow those outlined above except that the exposure time for the autoradiograms was cut to 6 weeks.

APPENDIX 3

Statistical Procedures[2]

3.1 ANALYZING DATA FROM THE LONG-SURVIVAL THYMIDINE AUTORADIOGRAPHIC SERIES (CHAPTERS 3 AND 11–15)

Throughout the quantitative analyses of the time of origin of neurons in the long-survival autoradiographic series, it was noted that trends in cell labeling within animals were very consistent. For example, in the late injection groups (E20+E21, E21+E22), the superficial layers of the primary visual cortex (OC1M and OC1B) had more labeled cells than were in the superficial layers of the medial secondary visual cortex (OC2M Chapter 11); however variability between animals in an injection group were large enough to mask this trend. Consequently, two different statistical procedures were used. The first, the sign test (Conover, 1971) is a nonparametric test to determine the consistency of paired observations ("X" and "Y") within a single experimental unit (the individual animals in each injection group). In the experimental design for the time of origin data, the proportion of labeled cells in the two locations that were being compared represented the X and Y values within each animal in an injection group. The comparisons are grouped into three categories: (1) $X > Y$, "−" comparison; (2) $X < Y$, "+" comparison; 3) $X = Y$, "0" comparison. The zero comparisons are discarded and, depending on the total number of remaining "+" and "−" comparisons, either a binomial distribution or a normal approximation is used to calculate probabilities (P). The second, the repeated measures analysis of variance (Winer, 1971), tests the consistency of sequential observations (for example, the proportion of labeled cells in different layers of the cortex) in a single experimental unit. Since there were unequal numbers of animals in the various injection groups (unbalanced design), the data were analyzed using the repeated measures analysis of variance in the General Linear Models procedure (Proc GLM) in the SAS statistical package.

3.2 ANALYZING DATA FROM THE QUANTITATIVE EMBRYONIC STUDIES (CHAPTERS 8–10)

A variety of statistical tests from the SAS package were used. In Chapter 8, the quantitative data were collected on equal numbers of animals in each age group (balanced design). Therefore, the cell packing density, nuclear area, and orientation data were analyzed using one- and two-way analyses of variance in the ANOVA procedure for equal numbers of observations in each cell. In Chapter 9, the data on the proportion of horizontally oriented cells in anterior versus posterior parts of the dorsal cortex (Fig. 9–13) were analyzed using a repeated measures analysis of variance (included in the ANOVA procedure) because the two different observations were done within the same animals. In Chapter 10, the data on the packing density of cells in various layers of the developing cortex between the control group (normal untreated embryos) and the experimental group (embryos killed 6 hours after exposure to 200 R x-rays) were analyzed using the regression procedure (Proc REG) to see if the slopes of the lines were different in the two groups.

[2] Throughout the statistical analyses, we acknowledge the advice of Dr. James A. Norton in the Department of Psychiatry, Indiana University School of Medicine in Indianapolis, IN, and Dr. George McCabe, Dr. Myra Samuels, and Laura Humphreys in the Department of Statistics, Purdue University in West Lafayette, IN.

APPENDIX 4

Quantitative Procedures for Three-Dimensional Computer Reconstructions

4.1 CHOOSING THE SPECIMENS AND PHOTOGRAPHING THE SECTIONS

The entire neocortex and a portion of the lateral limbic cortex was reconstructed in three dimensions from paraffin-sectioned embryos (part of our normal collection described above) on days E15, E16, E17, E19, and E21. Because of the lengthy procedures involved in each reconstruction, only one representative embryo was chosen from each age group. Depending on the age of the embryo, every other section (E15) or every third section (E16–E21) through the cortex was photographed at low magnification using a Leitz/Wild macroscope equipped with a zoom lens (6.3X to 35X) and an automatic exposure camera unit. The entire telencephalon and diencephalon on the right side of the brain, including the midline, was in each photograph. To assure that each section was photographed at exactly the same magnification, the zoom lens was marked at the magnification needed for the largest section, and all further sections were photographed at that magnification mark. A stage micrometer was also photographed at the set magnification at the beginning, midway, and at the end of the photographic session for each embryonic brain.

4.2 ALIGNING THE PHOTOGRAPHED BRAIN SECTIONS

In small embryonic brains, extensive tissue damage results from placing pinholes or lengthwise lesions that could serve as guidelines for alignment of the sections into a three-dimensional structure. We considered putting pinholes for orientation into the opposite hemisphere, but this requires taking the photographs at a much lower magnification so that fine details (such as the border between the ventricular and subventricular zones) are difficult to see. Consequently, the photographed sections were aligned using a different method. By examining midline sagittal and parasagittal sections of embryos of the same age, the height of the dome of the cortex as it curves over the diencephalon was determined. Starting with the photograph of the middle anteroposterior section of the cortex, at approximately the highest point on the dome, we marked three fiducial points so as to form a triangle; that section was named the *orientation section*. Using a light board, we transilluminated the photograph of the orientation section and placed the photograph of the section in front of it, matching up several anatomical landmarks. Once the section was aligned with the one used for orientation, we marked the fiducials on that photograph. Then, we removed the photograph of the orientation section and used the section that was just marked as a template to place fiducials on the next section in front. Periodically, we checked the fiducials of the photograph now being aligned with the fiducials on the orientation section to see how they conformed to the curvature in the sagittally sectioned brain. After sections in front of the orientation section were aligned, we used the same procedures to align the sections behind the orientation section.

4.3 DELINEATING THE NEOCORTEX AND COLLECTING THE RAW DATA

In order to visualize lamination in the neocortex from its medial edge, we did not reconstruct the medial cortical wall, and a line angling approximately 45° from the midline was drawn through the dorsomedial edge of the neocortex. To delineate the ventrolateral edge, a straight (or slightly curved) line was drawn from the most lateral extension of the neuroepithelium above the ganglionic eminence to the ventrolateral edge of

the cortical plate, taking care not to include any part of the striatum. Using a Rapidograph Pen (#00 point), we drew the outlines of the entire wedge of neocortex (within the defined borders) on each photograph, and we drew lines at the interface between the ventricular and subventricular zones, at the base of the cortical plate, and at the surface of the cortical plate. Those delineations allowed us to reconstruct the entire neocortex, the ventricular zone (neuroepithelium), and the cortical plate. Boundaries between the subventricular zone, intermediate zone, upper intermediate zone, and the subplate are not definite; consequently, these layers were reconstructed together as the cortical transitional field that extended from the superficial border of the neuroepithelium to the base of the cortical plate.

The marked photographs were placed onto a Summagraphics Digitizer pad interfaced to an IBM PS/2 Model 80 computer with an 8514 monitor (resolution 1024 × 780).[3] First, the fiducials were digitized, followed by successively digitizing the outlines of the entire neocortex, the ventricular zone, the transitional field, and the cortical plate. The computer stored the X, Y coordinates outlining the profile of each structure and displayed the drawn outlines on the monitor. If the outlines were not accurately digitized, the program was set up to re-enter data for a particular section. Besides storing the X, Y coordinates, the computer also calculated the area of each structure and stored that along with the section number, which was used to determine the Z coordinate. After all of the sections had been digitized, a separate segment of the computer program aligned the data from each section in the file using the fiducial marks. The volume of each structure was also calculated. The final result was a large file (close to two megabytes in the E21 brain) that would serve as the data base for the reconstruction of either the entire neocortex, or specific layers.

[3] The computer program for the raw data collection of the three-dimensional reconstruction project was written by James Simmons and by Xiaofeng Dai, a graduate student in the Department of Computer Science, Purdue University, West Lafayette, IN.

4.4 FINAL RECONSTRUCTION

The three dimensional images were visualized by a software package, Skandha, developed by Dr. J. Prothero and his coworkers at the University of Washington, Seattle.[4] The data from selected sections in the large raw data file were sent to a Silicon Graphics IRIS work station and were prepared for Skandha by a file conversion program (Mor2dat). We used the raster display option in the menu so that images were reconstructed with solid surfaces. Rotation of the image in the X, Y, or Z coordinates to any desired vantage point is quickly and easily accomplished by using Skandha's interactive format. The "sun" altitude, ranging from −90° to +90°, and "sun" azimuth, ranging from −180° to +180°, could be changed to bring out depth by using the light source to cast shadows on image surfaces. Especially helpful in reconstruction of the neocortex, Skandha also has a feature that allows deeper structures (shown at full opacity) to be viewed beneath or inside of superficial structures (shown at 50% opacity); there is also an option to make certain features completely transparent (0% opacity). In the images that we show in Chapters 2 and 9 (Color Figs. 1–8), the most satisfactory views of the neocortex came from using a sun altitude of +4° and a sun azimuth of −15°. By making the transitional field completely transparent, we were able to reconstruct the ventricular zone and the cortical plate in their exact spatial relationship to each other.

[4] We were told about Skandha by Dr. Ray Russo, Department of Biology, Indiana-Purdue University, Indianapolis, IN. Dr. Russo has been extremely helpful with the images that are illustrated in Color Figs. 1–8. Licensing agreements for those who would like to use Skandha should contact Dr. Jim Barrett, Director, Health Sciences Center for Educational Resources, Health Science Building SB-56, University of Washington, Seattle, Washington 98195.

APPENDIX 5

Quantitative Procedures for Determining Cell Packing Density, Cell Size, and Cell Orientation[5]

5.1 CELL PACKING DENSITY

We analyzed a mid-anteroposterior strip of dorsal neocortex in sagittal sections that showed the greatest anterior extent of the olfactory evagination. The ventricular zone is present throughout the entire observed time span (E14–E22). Since the subventricular zone, the intermediate zone, and the cortical plate are not distinct in the dorsal neocortex until E17, quantification of packing density in those layers began then. Cells were counted using a 40X objective and a 10 × 10 ocular grid that delimited a total area of 0.333 mm^2 at a final magnification of 625X. The total number of cells in each square millimiter was then calculated from the unit area counts. The data for each layer was analyzed using a one-way analysis of variance (SAS GLM procedure) and the differences between selected means was tested using Tukey's Studentized range at an overall alpha level of 0.05.

5.2 DETERMINATION OF NUCLEAR AREA

In order to investigate planar differences in the appearance of cells in various layers, the dorsomedial neocortex from two sagittally sectioned and two coronally sectioned brains from each age group (E13–E22) was studied. The dorsomedial neocortex was chosen because all layers are cut perpendicular to the pial surface in both planes. The sagittal section analyzed was the one through the greatest anterior extent of the olfactory evagination (visible from E16 on), or at a corresponding level on E13–E15. From E18 to E22, the coronal section analyzed was the most posterior one showing the decussation of the anterior commissure. On and before E17 (when the anterior commissure first crosses the midline), the middle coronal section between the frontal and occipital poles of the neocortex was analyzed.

High-magnification photographs (160X, Zeiss photomicroscope III with a 40X objective) of the entire depth of a strip of the dorsomedial neocortex from the base of the ventricular zone to the pial surface were prepared for each embryo. Figure 8–1 shows a strip of dorsomedial neocortex from a coronally sectioned E17 brain and Fig. 8–2, a strip of dorsomedial neocortex from a sagittally sectioned brain of the same age. The photographs were assembled in a montage so that the entire strip of neocortex was reconstructed at a final magnification of 790X. A line representing the vertical meridian perpendicular to the pial surface was drawn through the montage (heavy vertical line, Figs. 8–1 and 8–2), and indications at the borders of the montage delimited the approximate boundaries of the various layers. The ventricular zone was measured from E13 to E22; the subventricular zone, intermediate zone, subplate, and cortical plate were measured from E17 to E22, and finally the upper intermediate zone was measured from E20 to E22. After a microscopic reexamination of the photographed area, only definite nuclear profiles were outlined with a #00 point Rapidograph pen. The areas of each outlined profile (examples are shown in the right halves of Figs. 8–1 and 8–2) were measured using a Summagraphics digitizer interfaced to an IBM PS/2 Model 80 computer. Only total nuclear profiles were measured: either all profiles

[5] All of the computer programs used for these analyses were written by Xiaofeng Dai, Department of Computer Science, Purdue University, West Lafayette, IN.

in a given layer or, in cases when the number of profiles was greater than 100, a random sample of the total. The computer program calculated the area of each profile, the mean and standard deviation of all the profiles in each layer and rank-ordered the profiles into histograms of size classes in each layer.

5.3 DETERMINATION OF CELL ORIENTATION

The same computer analysis that generated the nuclear area data was also used to determine cell orientation. That was accomplished by digitizing the vertical meridian (heavy vertical solid line in Figs. 8–1 and 8–2) in each photograph before the cell profiles were traced; the vertical meridian was considered to be rotated exactly 90° above the horizontal axis, which was set at 0°. From the X, Y coordinates that outlined the profile of the cell nucleus, the computer program determined the two most distant points on the perimeter and drew a straight line between these points to define the long axis of each nucleus. Then, the program determined the degrees that the long axis rotated above the horizontal. The very few cells that were perfectly round were assumed to have rotated 90°. Rotations in each layer were calculated separately. The computer program sorted the data into histograms containing three orientation groups. The vertical group (V) contained cells with nuclei rotated from 60° to 90° above the horizontal, the oblique (O) group from 30° to 59.9° above the horizontal, and the horizontal group (H) from 0° to 29.9° above the horizontal. Next, the proportion of cells within an orientation group was calculated by dividing the number of nuclei in the group by the total number of nuclei measured in the layer. By representing the data as proportional, the differences between the number of cells in the various layers were eliminated so that the focus would be clearly directed to orientation shifts between layers.

APPENDIX 6

Using x-Irradiation to Determine the Positions of Vulnerable Cell Populations

Low-level x-irradiation (200 R) has been used to locate primitive migratory and mitotic cells in the developing nervous system (Altman et al, 1968; Altman and Nicholson, 1971; Bayer and Altman, 1974). If animals are allowed to survive for only a short time after exposure (6 hours), the pyknotic fragments of the cells destroyed by the irradiation are still present, and their positions indicate regions of cell proliferation and/or migration. In this book, x-irradiation is used to quantify the degree of immaturity of the various cellular layers in a dorsomedial section of the developing neocortex.

Embryos from Purdue-Wistar timed pregnant rats were used; the morning when sperm were found in the vagina was considered the first day of embryonic development (E1). To accurately locate the regions of immature and vulnerable cells in the developing neocortex, two or more pregnant females were exposed to a single dose of 200 R x-rays from a GE Maxitron unit (300 kVp; half value layer, 2.4 mm copper) between 9 and 10:30 A.M. on each embryonic day from E13 to E22. Six hours after exposure, the dams were deeply anesthetized with sodium pentobarbital (35 mg/kg), and the embryos were removed by Cesarian section and either the whole body (E13-E16) or the heads (E17-E22) were dropped into Bouin's fixative. At the same time, other groups of embryos at the same ages were removed from undisturbed dams and were treated the same way as the x-irradiated embryos. After 24 hours in Bouin's fixative, the embryos of both groups were transferred to 10% neutral formalin until they were embedded in methacrylate according to standard histological procedures. Serial sections (3 μm) were prepared in the sagittal, coronal, and horizontal planes. For the sagittally sectioned embryos, a section through the level where the olfactory bulb (or its primordium) has its greatest anterior extent was chosen for quantitative analysis. Counts of the total number of cells (control group) or of the surviving cells (experimental group) were made in a middle anteroposterior strip of the dorsomedial neocortex in the ventricular zone (neuroepithelium), subventricular zone, intermediate zone, and the cortical plate.