Bayer SA and J Altman, (1991) NEOCORTICAL DEVELOPMENT, Raven Press.

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.