Neurogenesis in the Adult Rat: Electron Microscopic Analysis of Light Radioautographs

Abstract. *Three-month-old rats were injected intraperitoneally with [^{3}H]thymidine (4.3 microcuries per gram of body weight) and allowed to survive for 30 days. Radioautography of 1-μm sections revealed labeled cells in the granular layers of dentate gyrus and olfactory bulb; these were confirmed as neurons by electron microscopy of reembedded 1-μm sections.*

Several previous radioautoradiographic studies have implied that postnatal neurogenesis in rodents continues until only about day 20 in the olfactory bulb and in the dentate gyrus of the hippocampus (*1*, *2*). In contrast, other studies have suggested that neurogenesis in these regions continues into adulthood (*3*–*6*). The latter results have not been generally accepted, however, probably for two main reasons: (i) the labeled cells appear to be small granule cells which, in light micrographs, cannot be distinguished with certainty from neuroglial cells (*2*, *5*); and (ii) thick paraffin sections were used which allows for the possibility that a grazed, labeled neuroglial cell could have caused an underlying unlabeled neuron to appear labeled (*6*). We now report that new neurons are indeed being formed in the adult rat olfactory bulb and dentate gyrus, substantiated by electron micrographs of [H^3]thymidine-labeled cells. These electron micrographs were obtained by a new method that allows serial thin sectioning of 1-μm, plastic embedded sections previously prepared for light microscopic radioautography.

Four 90-day-old male rats (Charles River) were injected intraperitoneally with [^{3}H]thymidine (4.3 μc per gram of body weight; New England Nuclear, 20 c/mmole). Thirty days later the animals were perfused through the heart with a solution consisting of 1 percent glutaraldehyde, 4 percent paraformaldehyde, 0.1 percent hydrogen peroxide (*7*), and 0.01 percent $CaCl_2$ in 0.1*M* cacodylate buffer and left in the refrigerator overnight. The next day slices (1 mm) of the entire right olfactory bulb, as well as portions of the ventral and dorsal hippocampus, were postfixed in 1 percent OsO_4, dehydrated in ethanol and propylene oxide, and embedded in Araldite 502. Sections (1 μm) were cut, mounted on glass slides, and dipped into Kodak NTB-2 emulsion at 40° to 42°C, exposed for 1 month at 4°C (*8*), and stained with 1 percent toluidine blue and 0.4 percent sodium borate.

Sections in which a labeled cell appeared to possess a long dendrite were lifted off the glass slide and reembedded for serial thin sectioning. Since only a few labeled cells appeared to have such dendrites, it was necessary to develop a method of removing 1-μm sections from glass slides that was totally reliable. The cap of a Beem capsule was removed, and the closed end was cut off to create an open cylinder. Partially polymerized Araldite (heated at 90°C for 20 to 30 minutes) was sparsely applied to the smooth rim at one end of the cylinder; this rim was then aligned around the section to be lifted off. After the interior of the cylinder was filled with Araldite, the slide and mounted capsule were placed in an oven to polymerize, either for 1 hour at 90°C or 9 hours at 60°C. When the Araldite was fully polymerized, the Beem capsule mold was cut from around it and the glass of the slide was broken to leave a small square beneath the cylinder. The cylinder was then placed at an angle to the side of a plastic beaker and submerged in full-strength hydrofluoric acid for 90 to 120 minutes. After the hydrofluoric acid was washed off with running water, the cylinder of plastic was mounted in a trimming block, and the edges of the glass, now considerably thinned, were gently lifted until the glass popped from the top of the cylinder, leaving the 1-μm section flat on the base of the cylinder of plastic. If left in the acid too long, the glass became too thin to lift off in one piece; in these instances the cylinder was plunged into liquid nitrogen for 30 seconds, and immediately immersed in an ultrasonic cleaner; this procedure effectively removed the glass sheet. After a second washing in running water the section was mesa trimmed to a small region that included the labeled cell, and serial thin sections were cut with a diamond knife and mounted on slot grids (*9*). Camera lucida drawings at 40 × and 100 × of the original 1-μm sections were used to identify the labeled cell in the electron microscope.

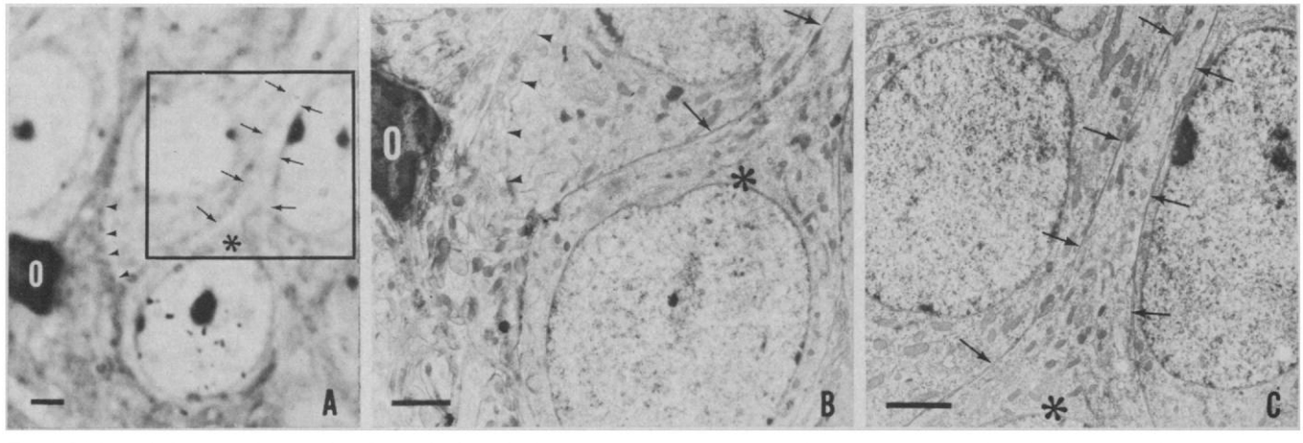

Fig. 1. Light and electron micrographs of granular layer in dentate gyrus; 4-month-old rat injected 30 days earlier with [^{3}H]thymidine. Scale bar, 2 μm. (A) Light radioautograph (1 μm section) of a granule cell (*) with numerous silver grains over its nucleus and an apparent dendrite (arrows) extending between two other granule cells. The very dark nucleus on the left belongs to an oligodendrocyte (O); small arrowheads indicate the edge of an unlabeled granule cell, also present in (B). (B) The same granule cell (*) labeled in (A) can be seen in this electron micrograph taken from the reembedded 1-μm section. Note homogeneous nucleoplasm, round contours to cell body, Golgi complex (just above indentation in nuclear envelope), and dendrite extending to upper right (arrows). Densely stained nucleolus in A is only faint in B because it does not extend throughout the thickness of the 1-μm section and only a portion of it is contained within this thin section. (C) Electron micrograph of dendrite (arrows) of labeled granule cell (*) shown in (A) and (B).

A cell was considered labeled if it had five grains over the nucleus, a value considerably above background (6 grains per 10,000 μm^2 or about 0.08 grain per cell). In 15 sections of the granule cell layer of the olfactory bulb, 615 apparent granule cells were labeled, and in 20 sections of the dentate gyrus 58 apparent granule cells were labeled. In addition, smaller numbers of labeled cells that appeared to be neuroglial cells were found, but these were not studied further. Thirty-five of the apparent granule cells were reembedded and examined in the electron microscope. These cells had features normally considered to be characteristic of neurons (*10*): (i) long processes, filled with a parallel array of microtubules, that resemble dendrites (Fig. 1); (ii) smooth contoured cell bodies (Figs. 1 and 2); (iii) homogeneous, light-staining karyoplasm of dentate gyrus granule cells (Fig. 1); (iv) symmetrical, Gray's type 2 synapses (Fig. 2) typical of axosomatic synapses previously described on olfactory bulb granule cells (*11*). These features, especially the dendrites and synapses, served to unambiguously identify these cells as neurons. Furthermore, the labeled cells did not resemble neuroglial cells, since they lacked the irregular contours of protoplasmic astrocytes and microglia, the bundles of filaments of fibrous astrocytes, and the electron-opaque cytoplasm and abundant heterochromatin of oligodendrocytes and microglia (*10*).

In cross sections of the entire dentate gyrus, there were between 0 and 8 labeled cells per section, and, of 58 labeled cells encountered, 12 were heavily labeled. A granule cell was considered heavily labeled if it had at least 19 grains over its nucleus (one-half the maximum number of silver grains observed over any granule cell). Preliminary examination indicates that there may be regional differences in the labeling of the granule cells in the dentate gyrus. For example, sections from the ventral hippocampus have, on the average, almost twice (1.9 times) the number of labeled cells in the dentate gyrus as those from the dorsal hippocampus. In addition, almost all the labeled cells in both regions seem to be present near the junction of the suprapyramidal and infrapyramidal limbs.

In the olfactory bulb, besides the many labeled cells found in the granule cell layer, labeled granule cells were observed in the periglomerular region and the mitral cell layer. The percentage of heavily labeled granule cells in the granule cell layer was calculated by estimating the total number of granule cells per unit area, multiplying this number by the total area of the granule cell layer examined to obtain a value of 49,000 granule cells, and then dividing the number of heavily labeled granule cells observed (15) to obtain a percentage of heavily labeled granule cells of about 0.03 percent. If these heavily labeled cells have only divided once after incorporating the label, the percentage of heavily labeled internal granule cells (0.03 percent) can be used to predict the expected growth rate in the layer. If we assume that new neuron production continues at the same rate from 3 to 24 months as observed at 3 months, a 55 percent increase in the number of granule cells would be expected (*12*), a value reasonably close to the observed increase (45 percent) in the volume of the granule cell layer from 3 to 24 months (*13*). Suggestive evidence has also been found that the hippocampus may continue to increase in depth in adulthood (*14*). But since the number of labeled cells in the dentate gyrus appears to be different for different regions (see above), we have not attempted to predict a specific volume increase from our hippocampal results.

Although the labeled cells examined in the electron microscope are neurons and not neuroglia, it is still necessary to show that the labeling really indicates new neuron production in the normal animal. Several questions need to be answered. (i) Could metabolically unstable DNA have incorporated tritiated thymidine? A careful autoradiographic study (*15*) has not disclosed any evidence for significant turnover of DNA in mouse Purkinje cells during the first 90 days of life. Furthermore, it has been reported that in the mouse there is no noticeable dilution of labeling in neurons for more than 700 days (*16*). Consequently, if any turnover of DNA occurs, it must be limited to very small segments of the DNA chain and cannot account for the heavily labeled cells seen in our study. (ii) Could neurons have incorporated [^{3}H]thymidine as they became polyploid? This is very unlikely since there are now serious reservations, based on technical considerations, about earlier claims that polyploidy exists in certain large neurons (*17*), and there has never been any suggestion that small granule cells are polyploid (*10*). (iii) Does thymidine injection induce DNA synthesis? The best answer to this question comes from studies which show that, even in rats uninjected with thymidine, the olfactory bulb in-

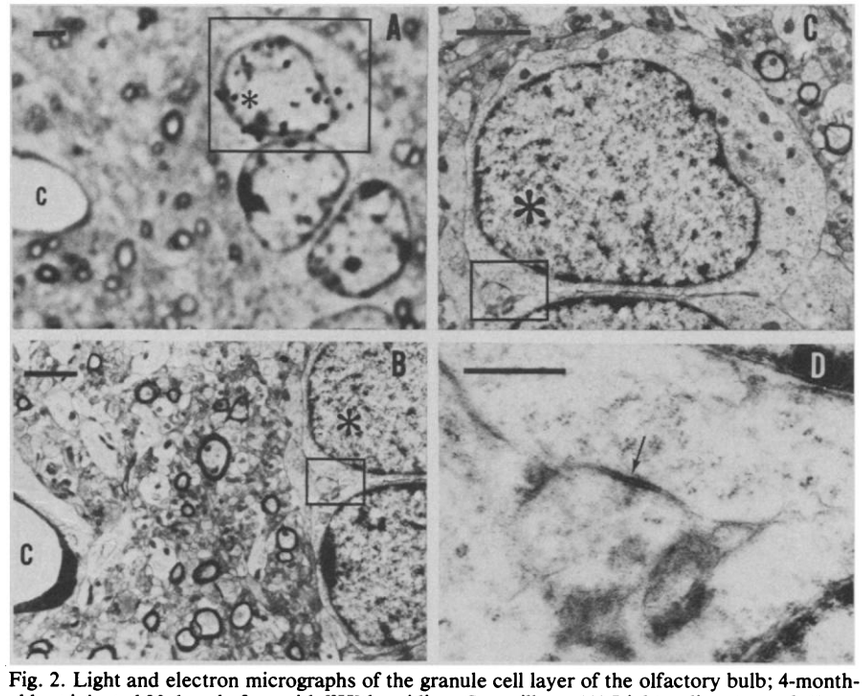

Fig. 2. Light and electron micrographs of the granule cell layer of the olfactory bulb; 4-month-old rat injected 30 days before with [^{3}H]thymidine; *C*, capillary; (A) Light radioautograph (1 μm section) of labeled granule cell (*). The box indicates the field shown at higher magnification in (C). Scale bar, 2 μm. (B) Electron micrograph of reembedded 1-μm section shown in (A). Capillary and myelinated axons are in the same position in both. A labeled granule cell (*) has a small axon terminal synapsing on its cell body; this region (in box) is shown at higher magnification in (D). Scale bar, 2 μm. (C) Cell body of labeled granule cell (*). Note the even contours of its cell membrane. The region of synapse onto the cell body (in box) shown in (D). Scale bar, 2 μm. (D) Higher magnification electron micrograph of axosomatic, Gray's type 2 synapse (arrow) on the perikaryon of the labeled granule cell (*). Scale bar, 0.5 μm.

creases in size during adult life (*13*), and the percentage increase in the size of the granule cell layer from 3 to 24 months is similar in magnitude to that predicted by the percentage of heavily labeled granule cells at 3 months (see above).

Thus, we conclude that the labeled granule cells observed in dentate gyrus and olfactory bulb of the adult rat represent newly formed neurons. A corollary of this conclusion is that the synapses found on labeled granule cells in the olfactory bulb must also have been newly formed in an adult animal.

These results indicate that the old concept that the adult mammalian brain is largely static is no longer tenable. Numerous studies have shown that experimental manipulations can lead to growth and plasticity in adult brain (*18*). Now we have confirmed that growth and plasticity, including neurogenesis and synaptogenesis, can also occur in the mature, unoperated, mammalian brain.

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12. The number of heavily labeled granule cells observed was 0.0307 percent of the total granule cell population (TGP) examined at 3 months or 0.000307 × TGP. The length of the S phase of subependymal cells [the probable source for late arising granule cells in the olfactory bulb (*4*)] in the adult rat has been estimated at 8.5 hours [P. D. Lewis, *Exp. Neurol.* **20**, 203 (1968)]. If we assume that the tritiated thymidine is available for incorporation into DNA for only a short time, less than an hour, 8.5 hours after injection almost all the labeled cells would have left S phase and another equally large population of cells could be labeled if another injection was given. This would then lead to a doubling of the number of heavily labeled granule cells seen in the olfactory bulb 30 days after injection compared with a single injection. On the assumption that neuron production continues at the same rate from 3 months (90 days) to 24 months (730 days), if one injected every 8.5 hours, the total number of heavily labeled cells that would be seen would be 640 days ÷ 8.5 hours = 1807 times the number of heavily labeled cells actually observed after the single injection at 3 months. The percentage increase in the number of granule cells from 3 to 24 months is therefore (0.000307)(TGP)(1807)(100)/TGP = 55 percent.
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An Adenylate Cyclase of Brain Reflects Propensity for Breast Cancer in Mice

Abstract. *High propensity for breast cancer in mice was associated with low dopamine-stimulated adenylate cyclase activity in the brain, low spontaneous motorization, and low motor responses to injections of the catecholamine precursor,* L-*dopa.*

Previous experiments showed that an increase occurred in the mean life span of mice consuming L-dopa (L-3,4-dihydroxyphenylanine) (*1*). This was ascribed to reduction of intervening diseases, for reasons which included the increased life span in a nonlethal affliction, Parkinson's disease, by L-dopa (*2*).

In Parkinsonism, improvement results from the following sequence. L-Dopa produces dopamine which stimulates an adenylate cyclase in postsynaptic neurons at dopaminergic synapses in the brain (*3*). This adenylate cyclase, discovered by Greengard *et al.* (*4*), is an important part of the dopamine receptor because it produces the adenosine 3′,5-monophosphate (cyclic AMP) which determines the responses of the postsynaptic neuron to dopamine.

Our initial studies and those of others (*5–7*) emphasized that this brain enzyme has behavioral roles because: (i) the cyclase was measured in the caudate nucleus which regulates motor behavior; (ii) it was modified by anti-Parkinson (*8*) and antipsychosis drugs (*9*) in vitro; and (iii) drugs which are normally used to change immunity, carcinogenesis, or protein synthesis have produced in intact mice quantitative correlations between the activity of the cyclase and the behavioral parameters studied earlier (*5, 6*). Nonetheless, a nonbehavioral role is suggested for this enzyme by (i) the cyclase extends beyond the caudate nucleus (*10, 11*) into areas which perhaps include the hypothalamus, a structure controlling hormonal function and metabolism, and (ii) feeding L-dopa to mice in amounts which usually increase life span potentiates the dopamine-stimulated cyclase (*12*). We suspected, therefore, that this enzyme might be a part of a system of the brain which registers or regulates the incidence of intervening diseases. To examine this hypothesis, we tested the cyclase in the brains of mice treated with immunotherapeutic materials and in the brains of untreated animals having a known propensity for a major disease, cancer.

The immunotherapeutic agents were given to groups of 36 female C3H/HeJ mice, 5 to 6 weeks old. One subgroup of 18 mice was injected intraperitoneally with BCG vaccine (1.5 mg in 0.5 ml of normal saline) (*13*) and another subgroup of 18 mice with normal saline solution once a week for 3 weeks. Similar subgroups were injected either with *Corynebacterium parvum* vaccine (1.4 mg in 0.2 ml of saline) (*14*) or with normal saline solution once a week for 3 weeks. The net dopamine-stimulated activities of the adenylate cyclase were measured after the addition of 200 μM of dopamine hydrochloride to the homogenates of the caudate nuclei as described (*8*). These activities, computed as picomoles of cyclic AMP per combined caudates of each animal were as follows: (i) BCG injected, 112 ± 14.8; controls, 59 ± 10.8 ($P <$ .01); (ii) *C. parvum* injected, 122.5 ± 4.4; controls, 54 ± 10 ($P <$ .01). The experiments with the *C. parvum* vaccine were repeated in their entirety on CD-1 male mice (5 to 6 weeks old) and C3H/HeJ breeder female mice (7 months old) with similar results.

In parallel experiments, mice were vaccinated with BCG or *C. parvum* and tested for their motor responses to intraperitoneally injected L-dopa (*6*). These