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MITOTIC NEUROBLASTS IN THE 9-DAY-OLD AND 11-MONTH-OLD RODENT HIPPOCAMPUS[1]

MICHAEL S. KAPLAN[2] AND DANNY H. BELL

Department of Anatomy, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

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Abstract

Ultrastructural identification of mitotic neuronal precursors beneath the basal hippocampal granule cell layer was made using electron micrographs of [^{3}H]thymidine-labeled cells. Ultrathin sections were obtained by a method that allows serial thin sectioning of reembedded sections previously prepared for light microscopic radioautography.

The electron microscopic observations reported in this study reveal: (*1*) that a steady rate of granule cell neurogenesis occurs during the first year of a rodent's life; (*2*) that newly formed granule neurons in the dentate gyrus of the newborn mouse and adult rat are a result of neuroblast division; and (*3*) two distinct classes of mitotic cells can be identified during the peak period of postnatal neurogenesis—those with synapses on their cell bodies and processes and those with no synapses or processes.

The classic concept that no new nerve cells are formed in the adult mammalian brain remains (Korr, 1980), even though there is abundant evidence that neurogenesis occurs after birth in mammals (Kaplan and Hinds, 1977; Graziadei and Monti-Graziadei, 1978; Kaplan, 1981), fish (Kirsche and Kirsche, 1961; Easter et al., 1977, 1981; Johns and Easter, 1976; Johns, 1976; Meyer, 1978; Birse et al., 1980), and amphibians (Gaze et al., 1979; Richter and Kranz, 1981). The controversy of whether fully formed neuroglia and nerve cells are incapable of multiplication (Schaper, 1894, 1897) or may divide (Hamilton, 1901; Hatai, 1901) began at least 100 years ago. Recent studies have probably not been generally accepted for two main reasons: (*1*) It is believed that neuroblasts do not have any mitotic potential in the mammalian brain (His, 1889; Caley and Maxwell, 1968; Boulder Committee, 1970; Jacobson, 1978; Korr, 1980; Schultze and Korr, 1981); and (*2*) previous investigations have suggested that large neurons may incorporate labeled DNA precursors due to metabolically unstable DNA or as a neuron becomes polyploid, not as a result of DNA synthesis (reviewed in Kaplan, 1981).

The terms "stem cell" or "blast cell" generally refer to relatively undifferentiated progenitor cells that have the capability of producing daughter cells, some of which undergo differentiation into the mature functional cells near their final position (Rakic and Sidman, 1968; Marin-Padilla, 1971; Sidman and Rakic, 1973; Rakic, 1975). Prior to differentiation, glial cells may undergo repeated mitoses (Paterson et al., 1973; Korr et al., 1975) with the last divisions occurring near the finial position of the cell (Hommes and Leblond, 1967; Das et al., 1974; Skoff et al., 1976; Basco et al., 1977; Mares and Brückner, 1978). Although recent studies have reported that advanced differentiated neuroglia may divide (Cavanaugh, 1970; Sturrock, 1975, 1976; Latov et al., 1979; Kaplan and Hinds, 1980; Sturrock and McRae, 1980; Reznikov et al., 1981; Sturrock, 1981), there have previously been conceptual obstacles that differentiated nerve cells or neuroblasts may divide. There are less strong objections to the division of other CNS cell types with processes and junctions during mitoses (Kaplan, 1980; Sturrock and McRae, 1980; Stevenson and Yoon, 1981; Sturrock, 1981). In fact, Gershon et al. (1981) reported mitotic enteric neuroblasts with phenotypic characteristics of mature neurons.

The concept of neurogenesis in the adult as documented by tritiated thymidine incorporation would be much less speculative if mitotic neuroblasts could be demonstrated by electron microscopy. Light microscopic studies of labeled granular neurons support the concept that hippocampal neurons may be formed in mammals just after birth (mouse, Angevine, 1965; rat, Schlessinger

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[2] To whom correspondence should be sent, at his present address: Ph.D.→M.D. Program (R-123), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101.

et al., 1975; mouse, Stanfield and Cowan, 1979; rhesus monkey, Rakic and Nowakowski, 1981). Serial survivals of animals injected with tritiated thymidine have demonstrated that shortly after injection mitotic and labeled cells are present in the base of the granule cell layer. With longer survivals, fewer of the basal cells are labeled and more granular neurons are labeled within the stratum granulosum (Angevine, 1965; Altman and Das, 1965b; Schlessinger et al., 1975; Cowan et al., 1980; Gueneau et al., 1982). Accordingly, the labeled neurons in the stratum granulosum have fewer silver grains over the nucleus than the precursor basal cells (Gueneau et al., 1982). Although these light microscopic studies have suggested that basal cells of the granule cell layer are neuronal precursors, there has only been one electron microscopic study, in the 2-month rabbit (Gueneau et al., 1982), to characterize the labeled cells after they left mitosis; e.g. 24 hr after injection of tritiated thymidine.

The present ultrastructural study was designed to quantify and characterize the number of labeled and mitotic cells in the rodent stratum granulosum to determine: (*1*) if unique neuronal precursors could be identified in either newborn or adult rodents and (*2*) how the rate of new neuron formation quantitatively changes during the first year of life.

Materials and Methods

Newborn mice. Seven mice (LAFIJ, Jackson Laboratories) were injected with 4 μCi of [^{3}H]thymidine (specific activity, 6 Ci/mmol) into the lateral cerebral ventrical at 9 days after birth using stereotaxic procedures. Several hours later (4 to 5.5 hr) the animals were anesthetized then perfused through the heart with a solution consisting of 1% paraformaldehyde and 4% glutaraldehyde in 0.08 M cacodylate buffer. Animals were stored in fixative for 12 hr, the brain was removed, and slices (1 mm) were cut perpendicular to the axis of the ventral hippocampal formation. Tissue slices were postfixed in 1% OsO_4, dehydrated in ethanol and propylene oxide, and embedded in Araldite. Twenty-five different series of serial sections (1.5 μm) were cut, mounted on glass slides, coated with Kodak NTB-2 emulsion at 40 to 42°C, exposed for 1 month at 4°C, developed and stained with 1% toluidine blue in 0.4% sodium borate. Mitotic and labeled cells were identified in the hippocampal subgranular zone using the light microscope. The number of grains over the nuclei was counted, and the location of each labeled cell was recorded. A granule cell was considered labled if five grains appeared over the nucleus. An average cell had 12 grains over the nucleus.

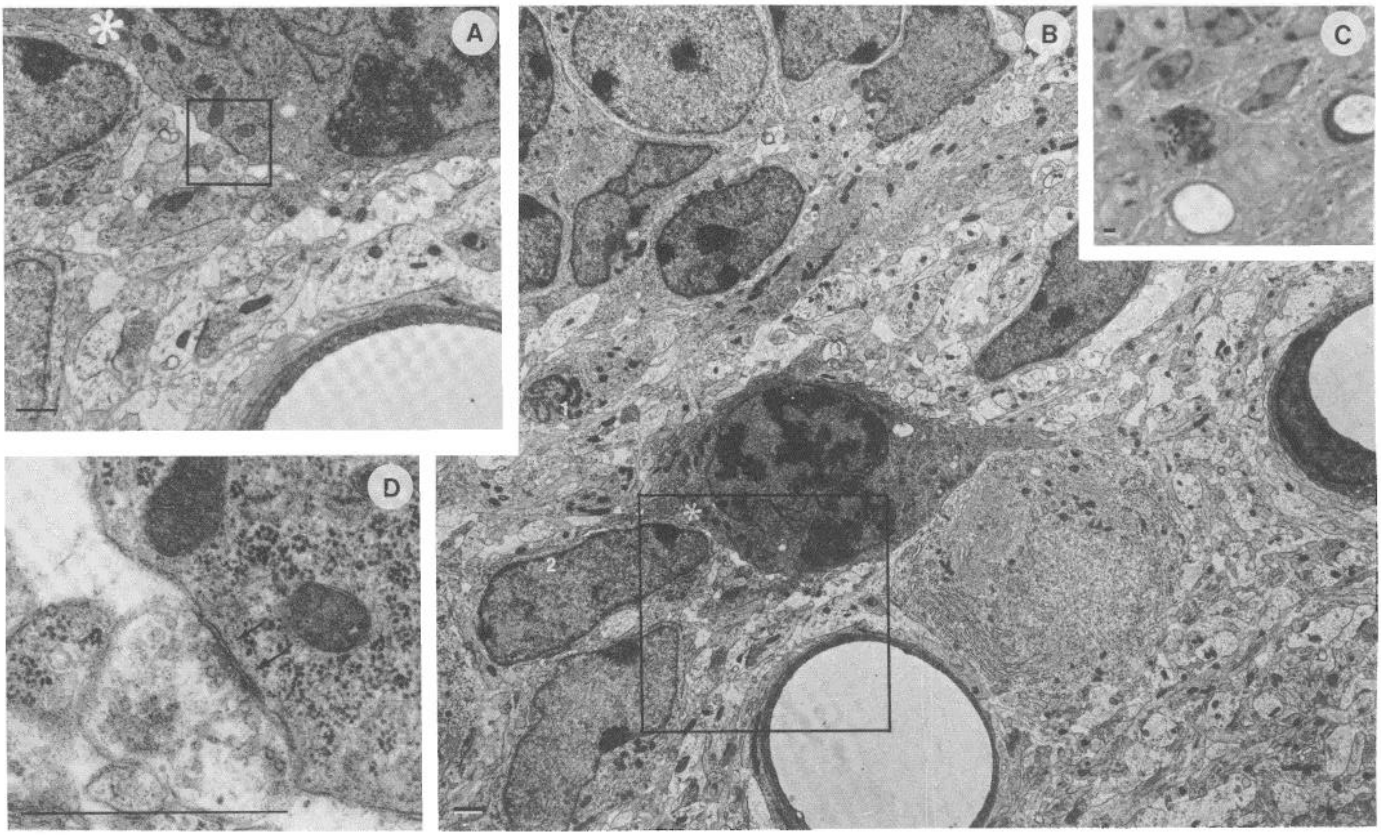

Figure 1. Mitotic cell in subgranular zone of the 9-day-old mouse hippocampus. The *asterisk* identifies this cell in Figures 1 and 2. *A*, Electron micrograph of a serial thin section through the *boxed region* shown in *B*. The labeled cell (*) has a small axon terminal synapsing onto the cell body (in the *box*) which is shown at higher magnification in *D*. *B*, Electron micrograph of the re-embedded labeled cell shown in the light microscopic radioautograph (*C*); the mitotic cell is labeled several hours after a single tritiated thymidine injection. For orientation between the light microscopic radioautograph (*C*) and the electron micrograph (*B*) of the re-embedded radioautographic section, note the two capillaries and adjacent undifferentiated cells in the same position. *D*, Higher magnification electron micrograph of axosomatic synapse (*arrows*) on the perikaryon of the labeled mitotic cell (*). *Scale bars*, 1 μm.

Each of three adjacent sections was serial thin sectioned for electron microscopic identification. Mitotic cells and labeled cells were randomly chosen for re-embedding. The procedure used to remove and re-embed the autoradiographic section from the slide was similar to the procedure described previously by Kaplan and Hinds (1977). The re-embedded section and slide were submerged in undiluted hydrofluoric acid for 60 min, then plunged into liquid nitrogen for 30 sec and imediately immersed in an ultransonic water bath. This procedure effectively removed the glass sheet over the re-embedded section and was faster than manually lifting the glass from the top of the section (Kaplan and Hinds, 1977).

All mitotic cells located in the re-embedded sections were examined in ultrathin sections (approximately 50) of the re-embedded serial 1.5-μm sections. The mitotic cells were identified in the subgranular zone—the area between the basal granule neurons of the granule cell layer and an irregular line connecting basket pyramidal neurons in the hippocampal hilus. The area examined was determined using a Ladd graphic data digitizer interfaced to a Monroe 1860 statistical programmable calculator.

Adult rats. Eight male (Sprague-Dawley) rats were injected with 5 to 7 μCi of [^{3}H]thymidine (specific activity, 52 Ci/mmol) into the lateral cerebral ventricle at 9 or 11 months after birth using stereotaxic coordinates. Twenty days later the animals were perfused, and tissue from the ventral hippocampal formation was saved. Technical procedures were identical to that described above, except serial 1.5-μm sections were not routinely processed. In the light microscope labeled cells were identified, the number of grains over the nuclei was counted, and the location of each labeled cell was recorded. After an entire section was examined, labeled cells were chosen for re-embedding and the 1.5-μm radioautographic section was lifted off the glass slide and re-embedded by the procedure described above.

Results

Newborn mice. All labeled cells were heavily labeled. This would be expected from the short survival interval (4 to 5.5 hr) after tritiated thymidine injection, which is enough time to observe the first cells entering mitosis (Sidman, 1970; Lewis, 1978; Korr, 1980). In light microscopic radioautographs, apparent "dark" stem cells were labeled in the base (subgranular zone) of the hippocampal granule cell layer. The percentage of mitotic cells (labeled and unlabeled), labeled mitotic, unlabeled mitotic, and labeled cells found in the subgranular zone was calculated by estimating the total number of cells (18, 711) in the area examined (6,596,809 μm^2). The number of labeled cells (1,024) then represented a 5.47% labeling index, and 50 observed mitotic cells were 0.267% of the identified cells. Most of these mitotic cells (0.214%) were labeled with silver grains over the nucleus 4 to 5.5 hr after tritiated thymidine injection. Sections containing two or more mitotic cells were re-embedded and thin sectioned for subsequent electron microscopic examination.

Most of the re-embedded mitotic cells had nuclear chromatin which appeared to be in prophase configuration prior to nuclear membrane disruption (Figs. 1 to 3). A few re-embedded cells had spine-like protrusions along the cell border which resembled those previously demonstrated on a mitotic cell in the 3-month-old rat visual cortex (Kaplan, 1981). Fifty percent of the 28 re-embedded mitotic cells in the newborn or adult granule cell layer exhibited axon terminals synapsing on the cell body or processes of these mitotic cells (Figs. 1, 2, and 4). Mitotic cells in late teleophase (Fig. 4) were observed with synapses on the cell bodies and on processes of these cells. Mitotic stages can be followed during the dissolution of the nuclear membrane and redistribution of chromosomal material accompanied by movement of cellular organelles to the periphery of the cell.

Gray type 1 axosomatic synapses were present (Figs. 5 and 6) (Laatsch and Cowan, 1966), but type 2 synapses were most commonly observed on mitotic cells (Figs. 1, 2, and 4). The type 1 axon terminals onto mitotic cells (Fig. 6) resembled mossy fiber terminals. Morphologically immature synapses were found on labeled cells (Fig. 3) which apparently had not entered mitosis. Centrioles were a regular feature of the interphase stage prior to mitosis (Hinds and Ruffett, 1971); the nuclear membrane is intact, and the interphase cell is heavily labeled. These early synapses (Fig. 3) were characterized by the presence of a postsynaptic density with few synaptic vesicles on the presynaptic membrane (Rees et al., 1976; Herndon et al., 1981; Jones, 1981; Kimmel et al., 1981; Robain et al., 1981).

Cells had processes which resembled neuronal neuritic growth cones (Pfenninger and Bunge, 1974; Rees et al., 1976). These processes (Figs. 3 and 4) contained agranular endoplasmic reticulum, many vesicles, and an occasional mitochondria. As Rees et al. (1976) described, large numbers of empty-appearing, smooth, round vesicles (varying diameter up to 200 nm) are clustered inside the plasma membrane which protrude as a mound up from the side of the cone (Figs. 3 and 4). Processes and organelles could be more completely reconstructed from a series of available re-embedded 1.5-μm sections than in single thin sections. Nevertheless, our partial reconstruction of these re-embedded cells could not demonstrate the full extent of the processes or organelles. Those cells which had long thin processes often had an obvious Golgi complex in the opposite neuroblast pole. These thin processes frequently contained free ribosomes and microtubules and resembled previous descriptions of immature axons (Figs. 1, 2, and 7). Proximal segments of processes were oriented tangentially to the long axis of the developing stratum granulosum, but some became radially oriented toward the granule cell layer (Figs. 1, 2, and 7) in distal segments. Unfortunately, many processes could not be traced beyond proximal regions. In this regard, a few processes resembled those from radially oriented migrating young hippocampal neurons (Nowakowski and Rakic, 1979).

Adult rats. Adult animals were sacrificed 20 days after tritiated thymidine injection. Therefore, the progeny of mitotic cells but not mitotic cells themselves, are labeled. In four 11-month-old animals, 52 apparent granule cells were labeled in 18 sections of the dentate gyrus. Labeled

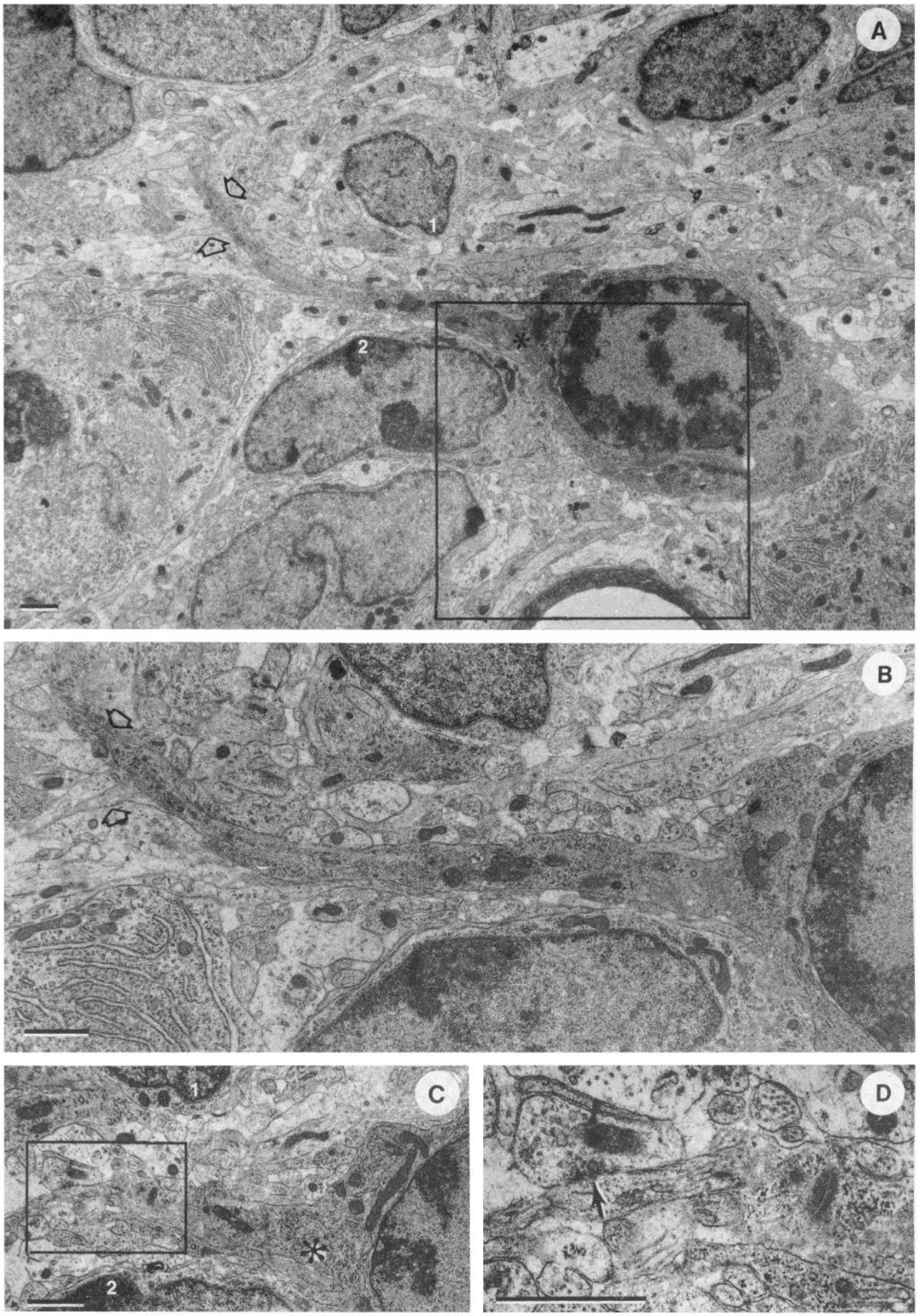

Figure 2. Electron micrograph of a serial thin section through the labeled mitotic cell shown in Figure 1. The *asterisk* identifies the labeled cell of the previous plate. *A*, Note the long immature axon (see the text) which courses between two undifferentiated cells numbered *1* and *2*. For orientation, these same cells are numbered in both plates. The *boxed region* in Figure 1*B* and the *boxed region* in Figure 2*A* are similar. *B*, Higher power electron micrograph of the process demonstrated in *A*; note the thin

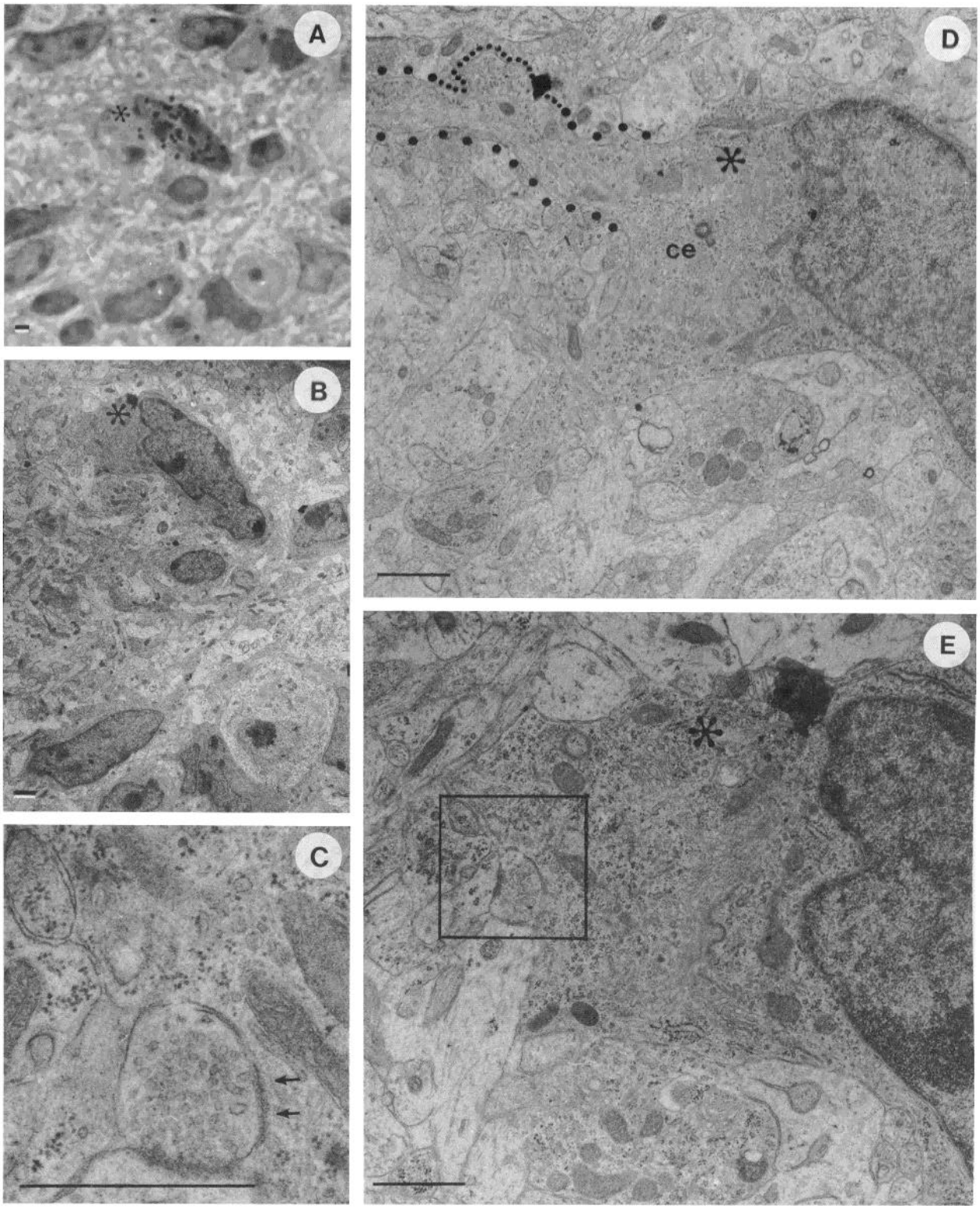

Figure 3. Premitotic labeled cell, several hours after tritiated thymidine injection into 9-day-old mice. The *asterisk* (*) identifies the labeled cell in the light microscopic radioautograph (*A*) and in the electron micrographs (*B, D,* and *E*) of the serial thin sectioned, re-embedded 1.5-μm section. *D*, Process (*dotted outline*) from the perikaryon of the labeled cell (*). *CE*, Centriole. The process displays agranular endoplasmic reticulum, occasional mitochondria, and free ribosomes clustered along and within the beaded shape process. *E*, Serial thin section of labeled cell (*). The *boxed region* is shown in *C*. *C*, High magnification through an adjacent thin section of the *boxed region* in *E*. Presumed immature synapse (*arrows*) has membrane densities and few vesicles on the presynaptic membrane. *Scale bars*, 1 μm.

microspike processes (*open arrowheads*). *C*, Adjacent serial thin section of immature axon. The labeled cell (*) process is located between previously identified undifferentiated cells (numbered *1* and *2*). The region containing a synapse (in the *box*) onto the immature axon is shown at higher magnification in *D*. *D*, Electron micrograph of apparent symmetrical synapse (*arrow*) on the immature process. *Scale bars*, 1 μm.

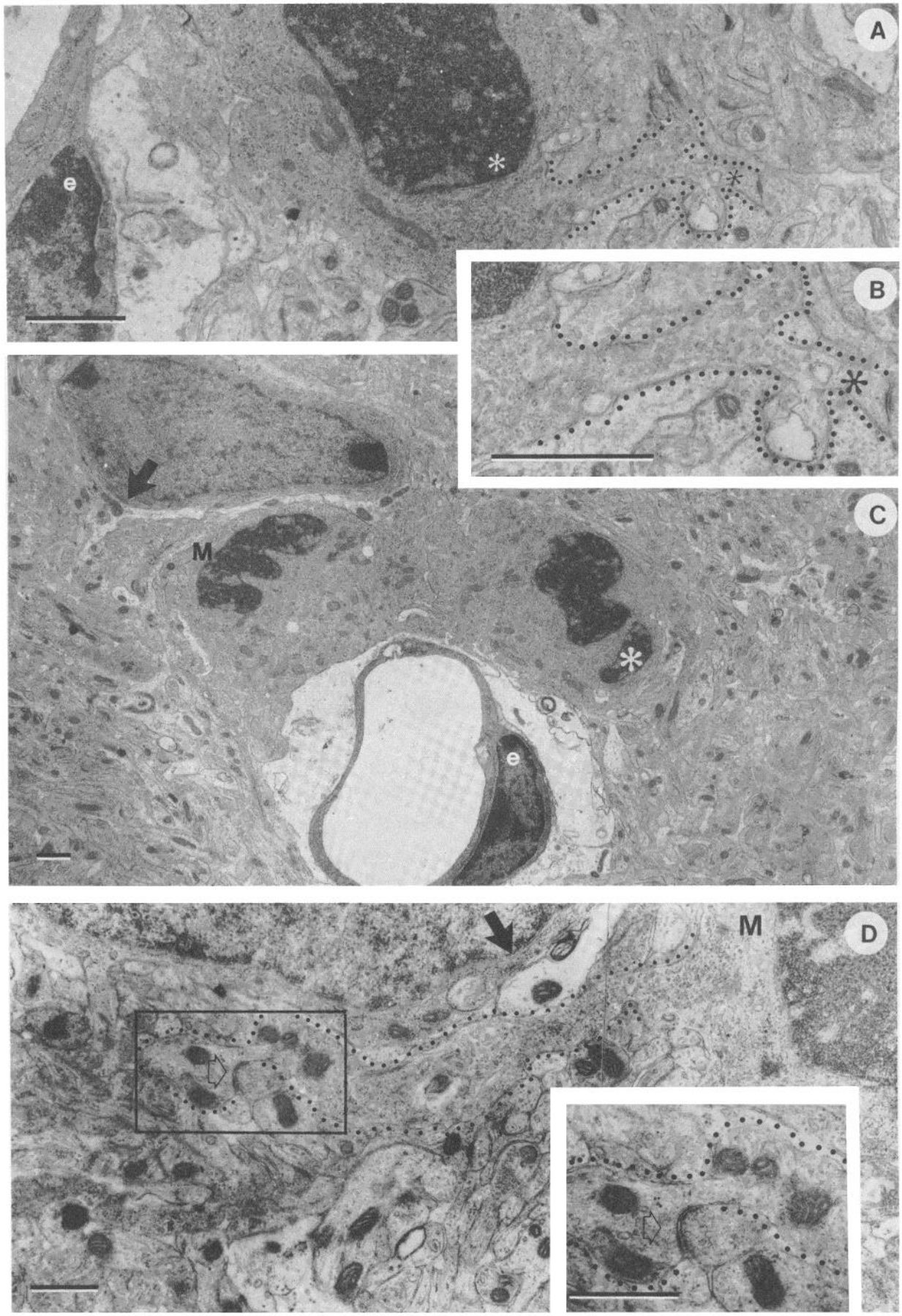

Figure 4. Mitotic cell in late teleophase, observed in the subgranular zone of the 9-day-old mouse. *A*, Future daughter cell (*) and process extending from cell body (*dotted line*). Process resembles neuronal neuritic growth cone (see the text), shown at higher magnification in *B*. *B*, Apparent growth cone with large numbers of round vesicles in the plasma membrane which

cells in adjacent 1.5-μm sections were examined to establish that labeling was not a result of random clustering of silver grains. Only cells with more than four grains over the nucleus (background—0.08 grain per cell) which were labeled in at least two adjacent 1.5-μm sections were chosen to be re-embedded for subsequent serial thin sectioning. Twenty-one of the labeled cells were identified as granule neurons with the light microscope. All were re-embedded. These re-embedded labeled cells were unequivocally identified as neurons by the presence of dendrites and synapses on the cell bodies and processes. The labeled neurons had features normally considered to be characteristic of granular neurons in the dentate gyrus (Laatsch and Cowan, 1966) (Fig. 5): (*1*) the nuclei had occasional shallow indentations; (*2*) nucleoli tended to be peripherally located; (*3*) uniformly dispersed chromatin occurred throughout the nucleus; (*4*) a narrow rim of cytoplasm surrounded the nucleus; (*5*) cisternae of the Golgi apparatus were distributed in the bases of the dendrites; (*6*) dense bodies were present; and (*7*) labeled granule cell perikarya were in immediate contact with neighboring cell bodies. Gray type 2 axosomatic synapses were present (Laatsch and Cowan, 1966), but type 1 axosomatic synapses were occasionally observed (Figs. 5 and 6). These type 1 axon terminals resembled mossy fiber terminals (Blackstad and Kjaerheim, 1961; Hamlyn, 1961; Laatsch and Cowan, 1966; Ibata and Otsuka, 1968).

Two mitotic cells were observed at the base of the dentate gyrus during light microscopic examination, and both were re-embedded and thin sectioned for electron microscopy. These mitotic cells are an indication of continued cell division about 1 month after injection of tritiated thymidine at 10 and 12 months after birth. In one animal which survived 10 months after birth, a mitotic cell had an early prophase configuration of chromosomes before nuclear membrane disruption (Fig. 6*A*). One remarkable feature of this mitotic cell (Fig. 6, *A* and *B*) is an axon type 1 terminal synapsing on its cell body.

Discussion

Extraventricular proliferation. The present study has demonstrated heavily labeled stem cells shortly after tritiated thymidine injections (Figs. 1 and 3) and lightly labeled mature cells 20 days after radionuclide injections (Fig. 5). However, the labeling intensity of the different cell types in the brain may not be uniform; apparently because there are differences in the incorporation of exogenous tritiated thymidine between cell populations (Kaplan, 1983). In the rodent, for example, glial cells seem to label more heavily than neurons (Sidman, 1970; Kaplan and Hinds, 1980; Kaplan, 1981). In fact, some dividing populations incorporate radioactive DNA precursors below background levels (Adelstein et al., 1964; Adelstein and Lyman, 1968). Perhaps the lower level of labeling found in the adult tissue might be due to an initially lower incorporation by mitotic cells compared to those in the neonates due to a more effective adult blood-brain barrier. Our observations of mitotic cells, labeled mitotic cells, and decreased grain counts with longer survivals support previous reports of differential uptake of DNA labeled precursors and indicate label dilution after subsequent divisions.

The demonstration of premitotic and mitotic cells with properties that characterize a specific cell type is not unique to cells of the central nervous system (erythroid cells, Holtzer et al., 1972; lymphocytes, Hesketh et al., 1977; choroid plexus, Kaplan, 1980; sympathetic neurons, Rothman et al., 1980; friend cell system, Levenson et al., 1980; pituitary, Saland, 1981). Other investigators have suggested that either differentiated or differentiating CNS cells *in vivo* may divide (Hamilton, 1901; Cavanaugh, 1970; Sturrock, 1975, 1976; Latov et al., 1979; Kaplan, 1981). Using electron microscopic radioautography, Reznikov et al. (1981) concluded that postnatal glial proliferation is independent from neurogenesis and that astroglia are capable of proliferation even in advanced stages of differentiation. Sturrock (1981) demonstrated with the electron microscope that oligodendrocytes which have commenced myelination are capable of undergoing mitosis without losing contact with their myelin sheaths, and Stevenson and Yoon (1981) have identified mitotic radial glial cells.

The fact that neurons can be induced to undergo DNA synthesis in culture (Geiger, 1957) by UV light or methyl methane sulfonate (Sanes and Okun, 1972) and by sustained depolarization (Stillwell et al., 1973; Cone and Cone, 1976) may be interpreted as supporting the concept of neuroblast division. It has been proposed (Cone, 1969, 1971; Cone and Tongier, 1971, 1973; Orr et al., 1972; Stillwell et al., 1973) that intracellular ionic concentration levels associated with electric transmembrane potential of cells may be functionally involved in cell division. A corollary of this hypothesis has been established in that lightly polarized nondividing neurons may become mitogenetic by depolarization—substantial increases in the level of intracellular Na^+ and decreases in K^+ (Cone, 1971; Stillwell et al., 1973; Cone and Cone, 1976). The synapses we have demonstrated on the cell bodies and axons of mitotic cells may perhaps serve as a key event in the initiation of neuroblast mitogenesis by depolarizing the postsynaptic membrane.

Characterization of neuroblasts. Numerous light microscopic studies have previously suggested that labeled and mitotic cells in the base of the granule cell layer in the newborn rodent are neuronal precursors (Altman and Das, 1965b; Angevine, 1965; Schlessinger et al., 1975;

protrude from the side of the cone. Note the puncta adherentia on this process and occasional agranular endoplasmic reticulum. *C*, Serial thin section of future daughter cells. For orientation between *A* and *C*, note the endothelial cell (*e*) and daughter cell (*) in similar positions. *D*, Serial thin section through other mitotic pole (*M*). For orientation, the *solid arrow* indicates similar positions in *C* and *D*. This pole (*M*) also has a process extending from the perikaryon (*dotted outline*). This growth cone-like process has a small axon terminal synapsing on it (*boxed region*) and is shown at higher magnification in the *lower right inset*. *Lower right inset*, higher magnification electron micrograph of symmetrical synapse with vesicles along presynaptic membrane. *Scale bars*, 1 μm.

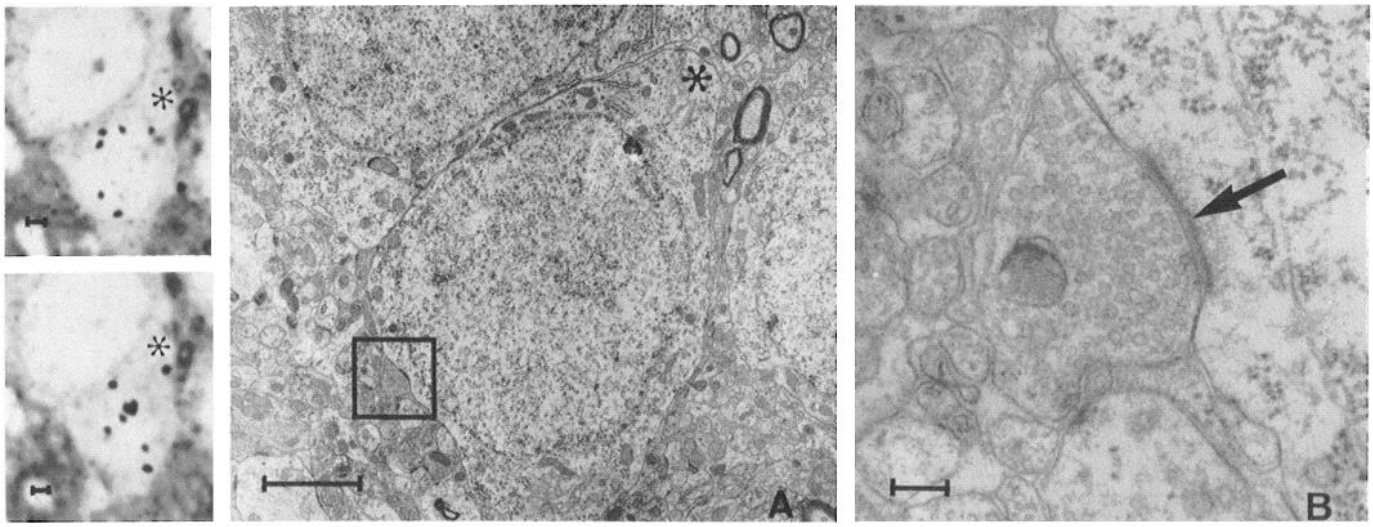

Figure 5. Neuron in the base of the granule cell layer of rat dentate gyrus; 290-day-old rat injected 20 days earlier with [^{3}H] thymidine. The *asterisk* identifies the labeled granule cell in two adjacent 1.5-μm light microscopic radioautographic sections (*left panel*; *scale bars*, 2 μm) and in the electron micrographs of the re-embedded 1.5-μm section (*A* and *B*). Adjacent unlabeled cells and myelinated axons are in similar positions in both light and electron micrographs. *A*, The same granule cell (*) labeled in adjacent 1.5-μm sections can be seen in this electron micrograph taken from one of the re-embedded 1.5-μm sections. The granule cell (*) has a small axon terminal synapsing on its cell body; this region (in *box*) is shown at higher magnification in *B*. *Scale bar*, 2 μm. *B*, Higher magnification electron micrograph of axosomatic, Gray's type 1 synapse (*arrow*) on the perikaryon of the labeled granule cell (*). *Scale bar*, 0.5 μm.

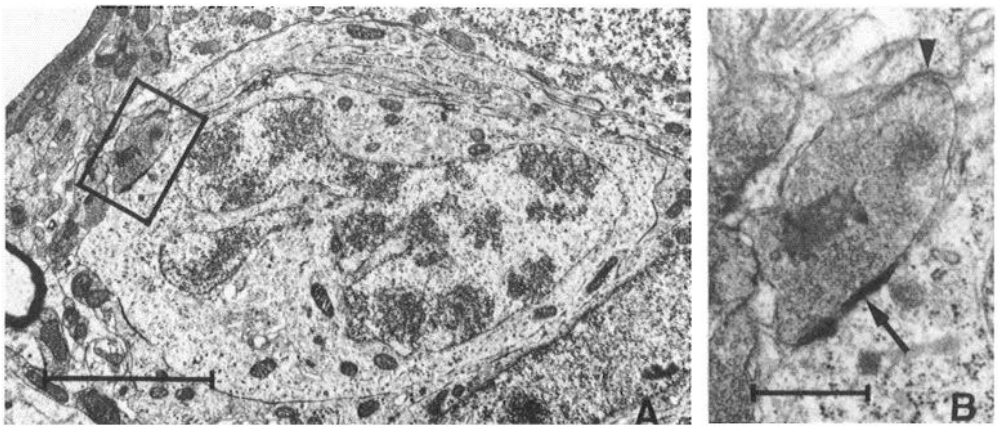

Figure 6. Prophase mitotic cell in the base of the granule of the dentate gyrus; 290-day-old rat. *A*, Electron micrograph demonstrates lumps of densely packed filaments in irregular shapes. Nucleoli could not be identified in serial thin sections of two re-embedded 1.5-μm sections. The region of a synapse onto the cell body (in *box*) is shown in *B*. *Scale bar*, 1 μm. *B*, Higher magnification electron micrograph of axosomatic, Gray's type 1 synapse (*arrow*) on the perikaryon of the mitotic cell. Note the dense packing of presynaptic vesicles and the presence of a second synaptic contact from the axon terminal (*arrowhead*). *Scale bar*, 0.5 μm.

Cowan et al., 1980). However, there has never been an electron microscopic radioautographic study to characterize these precursors (Leblond and Walker, 1956; Boulder Committee, 1970; Hinds, 1972a; Jacobson, 1978). We now report that the ultrastructure of granule neuron precursors in the postnatal dentate gyrus is not undifferentiated neuroepithelial cells but mitotic neuroblasts with synapses on their cell bodies and processes which resemble axons.

Previous electron microscopic studies on postnatal cell proliferation have not identified synapses or synapse-like contacts on mitotic cells (Mori and Leblond, 1969, 1970; Sturrock, 1974, 1981; Skoff et al., 1976; Kaplan, 1981) in the brain. Unlike the young 20-day-old neuron with an axosomatic type 1 synapse (Fig. 5), axosomatic Gray's type 2 synapses are typically found on granule neurons (Laatsch and Cowan, 1966; Ribak and Anderson, 1980). The type 1 axon terminals onto mitotic cells (Fig. 6) resembled mossy fiber terminals (Blackstand and Kjaerheim, 1961; Hamlyn, 1961; Laatsch and Cowan, 1966; Ibata and Otsuka, 1968) by dimension, the dense packing of presynaptic vesicles, agranular vesicles with wide range of diameters, electron density within the synaptic cleft, the presence of a single axon terminal making at least two separate contacts in a single section, and by a characteristic component of an area delimited by a single membrane which contained a wealth of synaptic vescles (Fig. 6).

Although typical symmetrical and asymmetrical synapses were found on the processes and cell body of mitotic

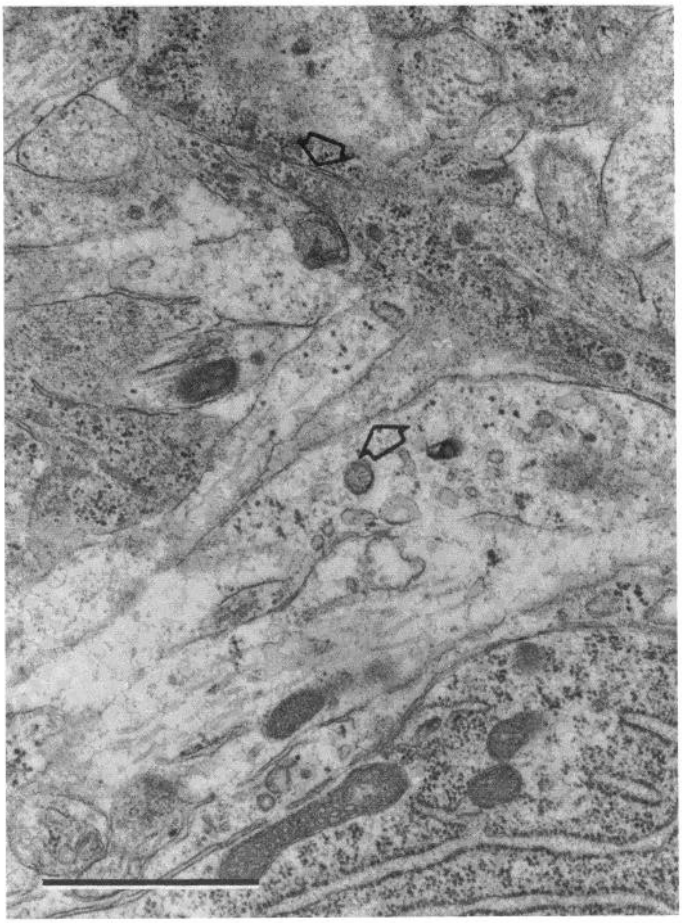

Figure 7. Distal portion of process in Figure 2*B*; higher magnification electron micrograph. Microspikes (*open arrowheads*) are obvious projections of the process. *Scale bars*, 1 μm.

cells, other investigations have suggested that "synapse-like" contacts may be present on mammalian glial cells (Grainger et al., 1968; James and Tresman, 1969; Henrikson and Vaughn, 1974; Wolff et al., 1979). These observations have been restricted to glial cell processes, not the cell body, of the prenatal animal. In fact, the presence of axoglial synapses is quite limited; they appear at E-11 and are not present after embryonic day 15 (Henrikson and Vaughn, 1974). These observations have been interpreted as errors in development (Henrikson and Vaughn, 1974; Wolff et al., 1979) or as regenerating axons which in culture cannot discriminate between neurons and glia (Grainger et al., 1968; James and Tresman, 1969). The formation of synapse-like contacts on glial cells may be demonstrated under very limited and special conditions and is not likely present in the postnatal hippocampus (J. E. Vaughn, personal communication).

Specific morphological characteristics such as the quantity of endoplasmic reticulum and the number of ribosomes can be recognized in neuroblasts (Fujita and Fujita, 1963; Meller et al., 1966; Wechsler and Meller, 1967). These cells do not resemble neuroglia cells, since they lack the irregular contours of protoplasmic astrocytes and microglia and the bundles of filaments of fibrous astrocytes (Peters et al., 1976). Neither do neuroblasts resemble immature forms of astrocytes or oligodendrocytes (Skoff et al., 1976; Imamoto et al., 1978; Kaplan and Hinds, 1980). Although the dissimilar features are outlined in Kaplan and Hinds (1980), it is interesting to note that immature neurons have an electron-dense cytoplasm (present study), unlike immature oligodendrocytes which have a light-staining cytoplasm. Conversely, mature neurons have a light-staining cytoplasm and mature oligodendrocytes are electron-dense (Kaplan and Hinds, 1980). Previous ultrastructural descriptions of the subgranular zone development have also observed distinct morphological transitions throughout gliogenesis (Reznikov et al., 1981; Gueneau et al., 1982). Morphologically distinct cells, including those with synapses on their cell bodies and processes and those with no synapses or processes, are labeled several hours after tritiated thymidine injection. Migrating postmitotic neurons have features similar to the neuroblasts described in our study (Nowakowski and Rakic, 1979). The nucleus is ovoid in shape, occasionally lobulated, and the cell has an electron-dense karyoplasm (Figs. 1, 2, 3, and 6). Our observations support the findings of Nowakowski and Rakic (1979) that neurons in more advanced maturation stages decrease in electron opacity (Fig. 5). It should be emphasized that no suitable cytochemical markers exist for immature neurons which are still undergoing migration and/or cell division (Marangos et al., 1980; Schmechel et al., 1980; Hawkes et al., 1982). However, neuronal precursors of the hippocampal dentate gyrus do not stain positively for glial fibrillary acid protein (Levitt and Rakic, 1980; Levitt et al., 1981). Our observations support the work of Levitt et al. (1981) on the coexistence of neuronal and glial precursor cells in the fetal monkey.

Our contention that mitotic neuroblasts may be identified after birth is supported by electron micrographs of synapses on apparently immature axons. Processes (Figs. 1, 2, and 7) had features normally considered to be characteristic of immature axons: (*1*) long processes which maintain a farily uniform caliber throughout their length (Spiedel, 1933; Hughes, 1953; Yamada et al., 1971; Hinds, 1972b; Peters et al., 1976), unlike immature axons most other processes begin as large cytoplasmic extensions of the perikarya (Caley and Maxwell, 1968; Shoukimas and Hinds, 1978) and contain more organelles (Caley and Maxwell, 1968); (*2*) neither fascicles of microtubules nor a dense undercoating of the axolemma were present on the proximal parts of immature axons (Hinds, 1972b; Shoukimas and Hinds, 1978); (*3*) axonal ribosomes predominate near the nucleus then become relatively indistinct in the distalmost part of immature axons (Hinds, 1972b; Shoukimas and Hinds, 1978); (*4*) filopodium-like processes "microspikes" are present on developing axons (Yamada et al., 1971; Hinds, 1972b); and (*5*) synapses were observed on processes (Figs. 2 and 4).

Neurogenesis in the adult hippocampus. The results of the present study, combined with those of previous investigators using similar techniques, allow the formation of several general statements regarding adult neurogenesis. First, there may be regional differences in the labeling of the granule cells in the dentate gyrus. For example, sections from the dorsal hippocampus of the 9-month-old animals contain half the number of heavily

labeled granular cells (Kaplan and Bell, 1983) as compared to the ventral hippocampus of 11-month animals (present study). Similar observations have been reported in animals injected with tritiated thymidine 3 months after birth (Kaplan and Hinds, 1977).

Second, it now appears that postnatal increases in granule cell layer volume may be partly a result of the proliferation of granule neuroblasts. Previous light microscopic radioautographic studies of thick paraffin sections have suggested that neurogenesis continued into adulthood (Altman, 1963, 1967, 1969; Altman and Das, 1965a, b). However, these labeled cells cannot be distinguished from neuroglial cells in sections greater than 1 μm thick with certainty (Altman, 1967; Ling et al., 1973; Kaplan and Hinds, 1980; Kaplan, 1981). After re-evaluation of the labeling in animals injected 3 months after birth (Kaplan and Hinds, 1977) it appears that approximately 0.044% of the granule neurons are heavily labeled 30 days after tritiated thymidine injection (12 labeled neurons per 27,097 granule cells). The percentage of heavily labeled granule cells (in the animals injected at 11 months after birth) may be calculated by estimating the total number of granule cells per unit area and multiplying this number by the total area of the granule cell layer examined (4.21×10^6 μm^2) to obtain a value of 24,387 granule cells; the percentage of heavily labeled apparent neurons in the granule cell layer is then about 0.045% (11 per 24,387). These values indicate that approximately 0.044% of the granule neurons appear heavily labeled 1 month after tritiated thymidine injection in either 3-month-old or 11-month-old rodents.

Third, equable labeling indices suggest that new granule neurons are being formed at a slow but continuous rate from 3 to 11 months after birth.[3] Therefore, the total number of heavily labeled cells that would be seen from 3 to 11 months would be: 240 days ÷ 11 hr (the length of DNA synthetic phase) = 524 times the number of heavily labeled cells actually observed after a single injection. The percentage increase in the number of total granule cell population (TGP) from 3 to 11 months is, therefore, (0.00044) (TGP) (524) (100)/TGP = 23% or 60% increase from 3 to 24 months (0.00044) (TGP) (1375) (100)(/TGP). Indeed, it appears that the number of newly formed granule neurons is appreciable during adulthood. These extrapolated increases of the number of newly formed granule neurons are very similar to the volume increases with age (Diamond et al., 1975; Bayer et al., 1982).

We conclude, based on electron microscopic analysis of light microscopic radioautographs, that the rapid rate of granule cell neurogenesis in the newborn[3] decreases to a low, but significant, steady rate during the first year of a rodent's life. Additionally, our ultrastructural observations of synapses onto the cell bodies of mitotic cells and labeled neurons demonstrate considerable morphological plasticity in the year-old rodent hippocampus and challenge the old concept that neuroblasts are postmitotic.

[3] In the newborn rodent the peak period of postnatal neurogenesis occurs within 3 weeks after birth (Angevine 1965; Bayer and Altman, 1974; Schlessinger et al., 1975; Lewis, 1978). Schlessinger and colleagues (1975) have estimated that about 50,000 granule neurons are generated each day between the fifth and eighth postnatal days. In our material we found a labeling index of 5.47 after a single injection of tritiated thymidine. The fractional increase in the number of granule cells in the 9-day-old mouse may then be estimated at 12% in 24 hr. With this labeling index one may attempt to assess the role of cell proliferation. The length of the DNA synthetic phase(s) has been estimated to be 11 hr for cells in the newborn dentate gyrus (Lewis, 1978). Since tritiated thymidine is available for incorporation into DNA for a fraction of S-phase (Sidman, 1970; Kaplan, 1983), at 11 hr after injection, almost all cells labeled would have left S-phase and another equally large population of cells could be labeled if another injection were given, doubling the number compared with a single injection. On the assumption that granule cell production continues at the same rate during the first few weeks after birth, one could calculate how many injections, if given once every 11 hr, are required for 100% of the cells to be labeled: 100%/5.4% = 18.52 injections, given every 11.0 hr. Thus, the granule cell precursors might double in population after 8 days (18.52 × 11 hr). These numbers confirm previous reports of a considerable growth to the population of granule neurons in the newborn rodent. This rapid rate of proliferation apparently decreases to a low but steady rate throughout adulthood.

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