THESIS

This is to certify that the

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A Golgi Study of Early Development in the Connate-Cuneate Nucleus Region of the Opossum

presented by

Steven Warach

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John I. Johnson
Major professor

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A GOLGI STUDY OF EARLY DEVELOPMENT IN THE CUNEATE-GRACILE NUCLEAR REGION OF THE OPOSSUM

By

Steven Warach

A THESIS

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ABSTRACT

A GOLGI STUDY OF EARLY DEVELOPMENT
IN THE CUNEATE-GRACILE NUCLEAR
REGION OF THE OPOSSUM

By

Steven Warach

The present work describes normal cellular development in a brain region involved in the processing and relay of somatosensory information. The cuneate-gracile nuclear complex of the opossum is known to be susceptible to modifications following early limb amputation. This study utilized Golgi impregnation techniques to examine normal cellular development throughout the period during which the documented effects of early peripheral injury occur.

Three stages of cellular development were observed.

1) Ventricular cells are classically mitotic or premigratory postmitotic cells. A number of these cells were observed in putative contacts with radial fibers from other cells. Two types of migration are proposed: Dorsal median ventricular cells are displaced laterally and probably depend heavily upon passive migration. Cells from the more lateral portion of the ventricular zone migrate by a nuclear translocation through their external radial processes.

2) Undifferentiated extraventricular cells are migrating or post-migratory neuroblasts.

3) Maturing cells are recognized as young neurons. Glia are not discernible by day 18.

Development in this region is gradual and does not occur in discrete phases. Cells at all three developmental stages are present throughout the 18 day period following birth. The dorsal outline of the nuclear region as well as incoming dorsal column fibers appear during days 4-6. Ventricular cells wane during synaptogenesis (7-9 days) and again following the end of cuneate's susceptibility to injury. The growth and differentiation of maturing cells are gradual and asymptotic throughout this period.

A discussion of the hypothetical mechanisms of nuclear volume loss induction in light of the present findings concludes that the hypothesis that nuclear loss results from a subnormal amount of cell proliferation is now the most promising lead. The triatiated thymidine experiments which would crucially test that hypothesis would also speak to many of the claims and questions raised in this study.

ACKNOWLEDGEMENTS

I wish to warmly thank Dr. John I. Johnson for his advice, guidance, support, encouragement, and faith. Drs. Glenn I. Hatton, Dennis A. Steindler, and Charles D. Tweedle made suggestions which have improved this thesis.

Stimulating discussions with E. Michael Ostapoff helped me make sense of much of the data.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Very little is known about the normal development of the dorsal column nuclei, a brain region involved in the processing and relay of somatosensory information. This matter is of particular interest because the cuneate-gracile complex (Cu-Gr) is susceptible to modifications following early peripheral damage (Johnson, Hamilton, Hsung & Ulinski, 1972; Schreck & Johnson, 1978). For example, removal of the distal forelimb from a 7 or 9 day old opossum results in a significantly smaller volume of the ipsilateral cuneate nucleus. This volume difference is present as early as postnatal 15. However, when the tissue is removed at postnatal day 15 a volume difference between the two sides cannot be detected (Schreck & Johnson, 1978). Amputation of an entire or partial hindlimb from a 4 or 5 day old, but not a 20 day old opossum results in the absence of that portion of the gracile nucleus with which the excised tissue would have been functionally connected, while the rest of Cu-Gr maintains its normal functional relationship to the remaining portions of the periphery (Johnson et al., 1972).

We have asked two main questions: (1) What developmental events between days 9 and 15 signal the end of the

susceptibility of Cu-Gr to peripheral lesions? (2) What are the cellular changes responsible for the Cu-Gr volume loss which follows early limb amputation? We have previously determined that nuclear volume loss is not the result of receptor removal, since specialized tactile receptors are not normally present in the glabrous forepaw skin until after 20 days of age, well beyond the end of the period of susceptibility to injury (Brenowitz, Tweedle, & Johnson, 1980).

The cellular changes induced by limb removal are unknown and still under investigation. An accurate assessment of the effects due to peripheral damage necessarily depends upon a knowledge of the details of normal development. Nissl and electron microscopic data of normal opossum Cu-Gr development have been generated (Ulinski, 1969; Tweedle, Hearshen & Ostapoff, 1977, respectively). The present Golgi study not only provides important complementary data regarding normal development, but also serves as a guide for future studies of the problem of how peripheral tissue influences Cu-Gr development. This study deals with animals up to 18 days old since by that age the documented changes due to the removal of limbs have taken place: the period of susceptibility to nuclear loss has come to a close and the nuclear loss is already apparent.

Nissl, myelin, and Golgi stained material from more mature animals were first examined, in order to gain an appreciation of the nuclear anatomy.

MATERIALS AND METHODS

Subjects. All neonatal subjects of known age were bred in captivity from adults trapped in the wild by us locally or by commercial firms in Texas or Florida. Pouches were examined daily at approximately the same time of day. The first day that young were discovered in the pouch was called day 1 of age. The exact age (± 24 hr) was thereby determined. Table 1 lists the distribution of subjects by age, litter, and type of Golgi impregnation. Nineteen animals from 5 litters were successfully impregnated. Most of the data will be from two litters having 5 and 10 subjects (litters a and b, respectively, in Table 1).

Golgi impregnated brains from nine more mature opossums were also examined. These specimens are also listed in Table 1. The age of these animals was approximated from snout-rump length (Reynolds, 1952; Ulinski, 1971) to range from 65-125 days. By 65 days all of the grossly visible adult structures of the brain have appeared (Ulinski, 1971), the Nissl cytoarchitecture throughout the rostro-caudal extent of the Cu-Gr has achieved its adult features (Ulinski, 1969), and well differentiated neurons with extensive dendritic patterns are present. Thus, while animals have not reached reproductive maturity by 65 days, neurons in Cu-Gr may be called adult.

Histological Techniques. The choice of the rapid Golgi technique to study developmental morphology is a logical one. It is acknowledged to be the most sensitive of the Golgi

techniques for young tissue. Ramón y Cajal attributed his unparalleled success in elucidating the nature of the nervous system to the use of his multiple impregnation rapid Golgi modification on embryonic and neonatal tissue (Ramón y Cajal, 1937, pp. 322-325). The work of Morest (1969, 1970) revealed delicately detailed rapid Golgi impregnations in the pouch young opossum. Therefore, the procedure most frequently employed in this study is based upon the perfusion rapid Golgi technique of Morest and Morest (1969). Brains from the first litter to be impregnated (n = 10) were cut into pre- and post-obex blocks. Staining parameters were varied for these 20 blocks and it was thereby determined that triple impregnations of 4-5 days in each of the chromate and silver solutions proved most successful. Subsequent impregnations were done following those parameters.

The animals were immobilized by hypothermia. The perfusing and impregnating solution for the first litter consisted of 3% potassium dichromate, 0.08M sodium cacodylate, and 0.5% osmium tetroxide in double distilled water. Immersion in 0.75% silver nitrate followed chromating. Subsequent impregnations used the exact solutions specified by Morest and Morest (1969).

For comparative purposes two other Golgi techniques were tried. A Golgi-Cox impregnation was obtained from an 8 day old animals (procedure of Ramon-Moliner, 1970). A specimen of 1 day of age was stained according to the Stensaas modification (1967) of the del Rio Hortega procedure.

Table 1.

Age (Days)	Subjects by litter
1	a, c, c, [1]
3	b
4	d
5	b
6	a, b
7	b
8	b, e[2]
9	b
10	a
11	b
12	a
13	b
15	a, b
18	b
65+	f[1], f[1], f[3], g[2], h[2], i[2], j, j, j[3]

Table 1. The number of subjects per age is listed according to litter with information on the type of impregnation. Each letter symbolizes a different litter. All impregnations were rapid Golgi except for those animals having superscripts 1 (Stensaas modification), 2 (Golgi-Cox; Ramon-Moliner), or 3 (Golgi-Cox; Anderson et al.). All neonatal ages are exact. The snout-rump lengths for the older specimens range from 108-250 mm.

Blocks were embedded in celloidin, except for the 3 day old specimen which was embedded in durcupan (Fluka A.G.); and sectioned transversely through the medulla at 60/120 μm, 80 μm, or 100 μm, except for the 8 and 13 day rapid Golgi specimens: the former was cut obliquely between the sagittal and horizontal planes and the latter between the transverse and horizontal planes. Nissl observations were made on brains prepared for a previous study (Ulinski, 1969).

Localization of cells. The dorsal boundary of Cu-Gr is not always discernible in Golgi impregnated sections. Nissl counterstaining with thionin or cresyl violet was usually performed on alternate sections (often following stabilization of the precipitate by the method of Geisert and Updyke, 1977) to assist in localization of Cu-Gr cells. Unfortunately, the counterstain was not very effective on the rapid Golgi sections, but normal Nissl stained material from comparable ages available from a previous study (Ulinski, 1969) was used as an aid in identifying Cu-Gr. For specimens in which cells are commonly along the ventricle, only those along the dorsal half of the alar plate were included. For deciding other ambiguities in the extent of the nuclear region the rule adopted was to be as conservative as possible (excluding any cells of doubtful identify).

All sections including presumptive Cu-Gr were microscopically examined. Over 500 Golgi impregnated cells from the neonatal animals were examined in detail.

The drawings presented were taken from a number of adjacent sections of one specimen at the specified age. In an

effort to minimize bias in the selection of cells presented, we drew all cells present in any section contributing to the sample of drawn cells. The one exception is the 18 day specimen; all the Cu-Gr cells from one half of one section were drawn. Drawings were made through x12.5 oculars, x40 (n.a. 0.65), x63 (n.a. 0.90), or x100 oil (n.a. 1.65) objectives, and a x12.5 ocular attached to a drawing tube.

RESULTS

Mature Specimens

Our observations of Nissl-stained sections confirm Ulinski's conclusion (1969) that the opossum Cu-Gr does not have a true cell cluster region. However, as Figures 1b and d illustrate, groups of 2 or 3 cells are often clumped closely together. Therefore, in contrast to the dramatic appearance of cells clusters and subnuclei in primates and carnivores (cf., Ramón y Cajal, 1952; Johnson, Welker & Pubols, 1968; Albright & Haines, 1978; Albright, 1978) the opossum "cell cluster" region (if that term has meaning for this species) is quite inconspicuous. Golgi impregnated material also reveals no morphological subdivisions in this middle region of Cu-Gr where clusters of tufted cells would be expected.

The cells of Cu-Gr do not easily fall into discrete morphological types as, for example, cells of the cerebellum do. What we see are gradations of morphologies out of which four relatively common, contrasting examples can be described (Figure 2).

(A) Bipolar cell. This type has a dorsoventrally oriented, round-oval soma measuring about 12 x 16 μm. Its two dendrite trunks usually give rise to 2 or 3 branches. The dendrites display smooth contours which are distally interrupted by a few lonely spines. This cell is most prevalent in the central rostro-caudal third of the nuclear region, the quasi-cluster zone (cf., Figure 1d). The bipolar cell may be the opossum homolog of Cajal's tufted cell (Ramón y Cajal,

1952). (B) Bouquet cell. The distinguishing feature of this cell type is the restriction of its dendritic origins and arborizations to one side of the cell body: the side facing the center of the nucleus. The soma is ovoid and may be either small (long diameter <16μm) or large (long diameter >20μm). The cell body is most often situated along the perimeter of the quasi-cluster zone. One thick or two thinner trunks branch into many smooth dendritic segments. This cell dominates in the caudal third of Cu-Gr. (C) Elongate cell. This is a large (long diameter >20μm) ovoid cell. Two or three dendrites emerge from its body. Each process may branch 1 or 2 times, but does not always do so. These cells are commonly found around the perimeter of the central portion of the nuclear region and throughout the pre-obex portion. No consistent somatic orientation is displayed. The dentrites tend to occupy the periphery of the nuclear region. (D) Stellate cell. This cell has a round soma. It may be small (diameter <16μ) or large (diameter >20μm). It typically sits in the middle of the nuclear region. The cell radiates 4 to 6 sparsely branched dendrites. These processes have a craggy contour but are generally aspiny.

Dendritic length is typically 100-300μm, although it may reach 750μm in larger cells. Axons emerge laterally and ventrally to comprise the internal arcuate fibers. Local circuit axons have not definitively been observed.

Afferent axons from the dorsal columns are approximately 1μm thick. Many bifurcate at their dorsal entry into the nucleus. The axon descends to the botton of the nucleus then

turns dorsally, giving off collaterals along its ascent. Collaterals spread in dorsoventral, mediolateral, and anteroposterior directions. Thin tortuous processes with irregularly shaped bulbous terminals densely innerviate the central portion of the nuclear region. The fine terminal bushes appear predominantly over dendritic rather than somatic regions.

Figure 1. Adult Cu-Gr. Photomicrographs a and b illustrate pre-obex sections through Cu-Gr in a myelin and Nissl stain respectively. The Nissl stained section is approximately 500 μm more anterior, at the rostral tip of the gracile nucleus. Adjacent sections through the most prominent portion of the nuclear complex (post-obex) are illustrated in c and d. Note that cell clusters are not a prominent feature of the opossum Cu-Gr. The Nissl stained sections have been magnified 1.25 times more than the myelin stained sections. Section thickness is 25 μm. Bar equals 250 μm. Dorsal is up. Lateral is to the right. M- median accessory nucleus of Bischoff, C- cuneate nucleus, G- gracile nucleus.

Figure 1.

Figure 2. Examples of adult cell types. A- Bipolar cells. B- Bouquet cell. C- Elongate cell. D- Stellate cell. Bar equals 50 μm. Arrows point to putative axons.

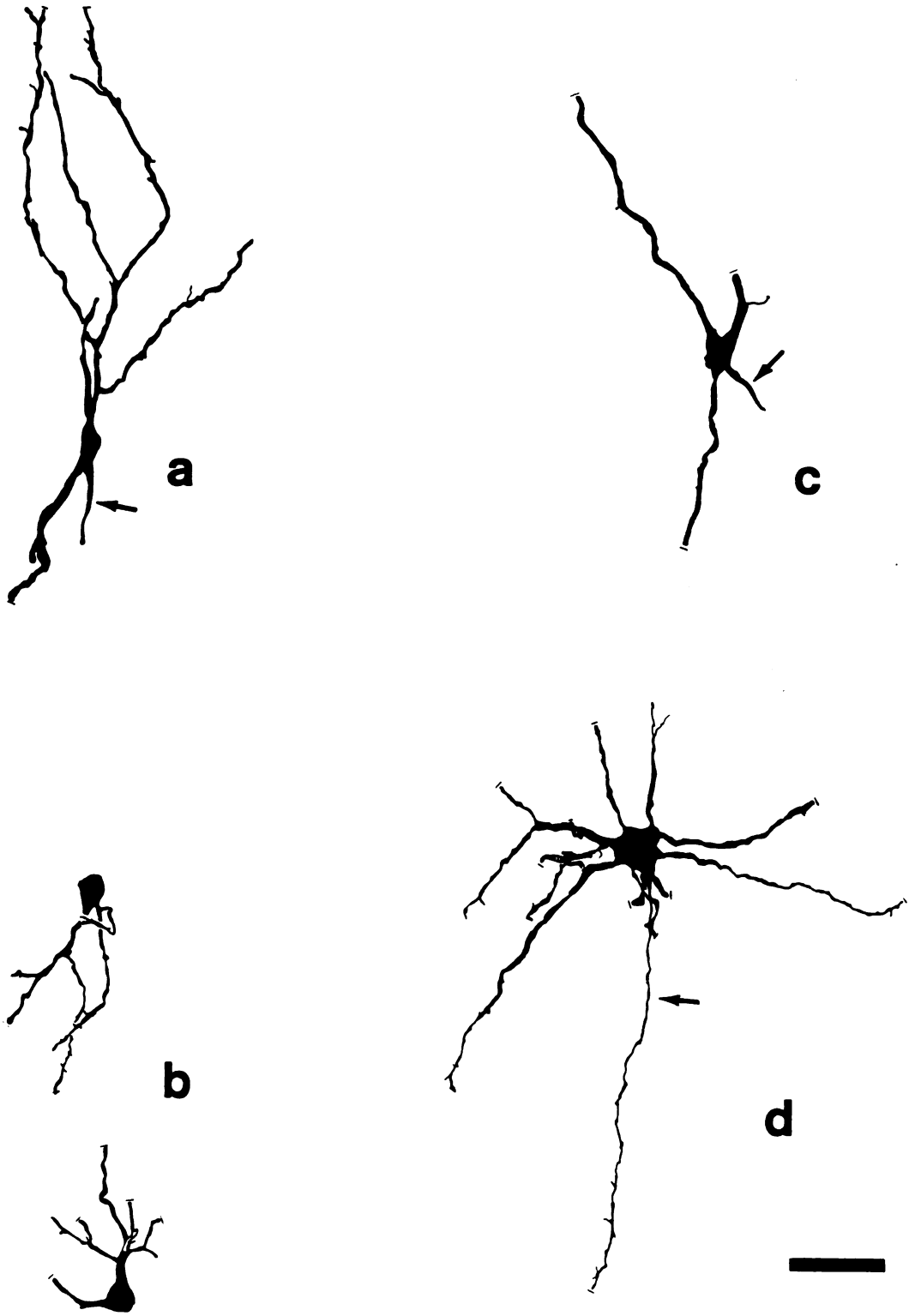

Figure 2.

Postnatal Days 1 through 18

The cuneate-gracile nuclear region develops gradually over the first 18 days following birth. The region is far from being mature by the end of this period. Cells have been morphologically categorized into three immature developmental stages. Throughout the first 18 days cells at each developmental stage are present in Cu-Gr. The categories defining each developmental stage are ventricular cells, undifferentiated extraventricular cells, and maturing cells. Examples of these cell types throughout the period in question are displayed in Figures 3-9.

Ventricular cells. In Nissl stained material an area of densely packed, deeply stained cells surrounds the ventricle. This zone extends from 75-100μm away from the ventricle. In rapid Golgi sections as well this ventricular zone is outlined by a darker background. Ventricular (V) cells lie within this zone. They are distinguished by morphology as well as position. The long axis of the V cell is perpendicular to the adjacent wall of the ventricle. Cells along the midline are therefore oriented dorsoventrally, while those along the lateral wall of the ventricle lie mediolaterally. The V cell has a bipolar ovoid soma approximately 6x10μm. Typically, it has a short process which extends toward the dorsal pial surface. The external process of the mediolaterally oriented V cell begins its course laterally and curves dorsally. It sometimes branches into thinner processes near the pial surface. The external process is believed to describe the path

of the cell's eventual migration. Roughly contoured varicosities and filopodia are commonly displayed by these processes. Occasionally, varicosities will apprear unusually large, irregularly shaped, and semi-translucent (e.g., Figure 5, 8, 11). These are reminiscent of the sheet-like outgrowths described by Peusner and Morest (1977) in the developing nucleus vestibularis tangentialis of the chick. Opaque inclusions are peppered inside such a varicosity.

Some V cells apparently have no internal process and abut the ventricular wall. Others may not show an external process.

Cells along the dorsal midline are considered to be V cells even in the special case in which their somata lie outside the ventricular zone. This exception is made because in no case has an extraventricular cell body in the more lateral portions of Cu-Gr been seen with an internal process ending in the ventricular zone. Dorsal median V cells have been observed as late as 90 postnatal days (snout-rump length 185mm).

The somata of V cells are often partially translucent (e.g., Figs. 4, 5, 8). The translucence usually appears as an oval patch, which suggests an unimpregnated nucleus surrounded by scant cytoplasm. However, the oval patch is sometimes interrupted by a bar of opacity, giving the dubious illusion (which one cannot fully appreciate in a two-dimensional representation) that the external process is passing through or under the soma (e.g., Figs. 3, 4, 5).

The V cell is the classic germinal cell: a mitotic cell or postmitotic cell which has yet to migrate away from the

ventricular zone to become a neuron or glial cell.

Undifferentiated Extraventricular cells. The undifferentiated extraventricular (UXV) cell is the migrating or undifferentiated post-migratory cell. The basic distinction between the V cell and the UXV cell is somatic distance from the ventricle. In the simplest case the UXV cell looks like a displaced V cell: a bipolar ovoid with no more than two roughly varicose processes. Typically, the UXV cell has no internal process, but exception can be found. No internal processes of UXV cells were observed ending in the ventricular zone. More developed UXV cells may have smoother, branched processes (e.g., Figure 6). Some UXV cells have little more than a soma (Figs. 4, 9).

Maturing cells. The morphological distinction between UXV cells and maturing (M) cells is somewhat ambiguous. At that fuzzy border are mono- or bipolar ovoids with varicosity-sparse unbranched processes. At the clearly differentiated extreme are M cells closely resembling adult neurons in their morphology. Occasionally M cells are seen within the ventricular zone (e.g., Figs. 6, 8). Axons of M cells are not readily identifiable throughout the 18 day period.

These three morphological categories of developmental stages have their counterpart in three types of Nissl stained cells (Figure 10A). The prominent cell of the ventricular zone is a deeply stained bipolar ovoid measuring about 5x6μm in paraffin-embedded sections and oriented perpendicularly to the ventricle wall. A similarly stained cell, the UXV cell,

is common outside of the ventricular zone. A third type of cell appears with a round nucleus, scantily stained karyoplasm containing a nucleolus and clumped chromatin, and little if any Nissl staining in the cytoplasm. Compare this Nissl M cell with the adult Nissl stained cell (Figure 10B). The adult cell contains prominent Nissl bodies in the cytoplasm and a nucleolus amidst an otherwise clear karyoplasm. These V, UXV, and M cell categories in Nissl gain validity by the fact that as age increases the number of V cells decreases and the number of M cells increases. Furthermore, as with the Golgi categories, all three types are present throughout the 18 day period and adult cells are not. In addition, an occasional Nissl stained M cell is seen in the ventricular zone, just as an occasional Golgi impregnated M cell is (cf., "Maturing Cells" above).

Figure 3-9. The following figures show examples of Golgi-impregnated neurons in single specimens throughout the three day periods which should be viewed side by side. The general pattern of the figures is as follows:

Upper left: A photomicrograph of a Nissl stained section sits next to a drawing of the section. The section corresponds to the approximate region from which the illustrated Golgi-impregnated neurons were drawn. The accompanying diagram demarcates the dorsal boundary of the nuclei (where present) and indicates presumptive Cu-Gr cells.

Lower left: Composite drawing of several Golgi-impregnated cells depicted in the arrays on the right hand page. Cells were drawn from both sides of the ventricle, but all are depicted on one side, i.e., some were rotated 180° around a dorsoventral axis. The approximate location of all cells relative to one another and to the dorsal and ventricular surfaces have been preserved.

Right: On this page, except for Figure 7, the entire sample of drawn Golgi-impregnated cells are listed according to developmental category. To avoid bias toward a particular cell type, all cells present in a section contributing to a sample were drawn.

Generally, ventricular cells are transposed to show their orientation to the ventricular wall, which is transposed into an invisible verticle line on the page. The undifferentiated extraventricular cells and maturing cells have been reoriented such that a verticle line through them would be normal to the nearest pial surface.

Short bars perpendicular to the end of the processes indicate that the fibers have been cut out of section or have been lost in a dense precipitate. Stippling indicates sheet-like outgrowths. All scale bars equal 50 μm. Dorsal is up; lateral is to the right.

Figure 3. Example of 3 day old, post-obex. A sample of cells caudal to the obex during the 1-3 day period. 1 - ventricular cell abutting the ventricular wall with no internal process. 2 - ventricular cell with opaque bar visible through a translucent soma. 3 - undifferentiated extraventricular cell with a sideward (ventral) origin of its radial fiber. 4 - maturing cell similar to bouquet cell in morphology. 5 - maturing cell similar to elongate cell in morphology. 6 - maturing cell similar to bipolar cell in morphology. V - ventricular zone.

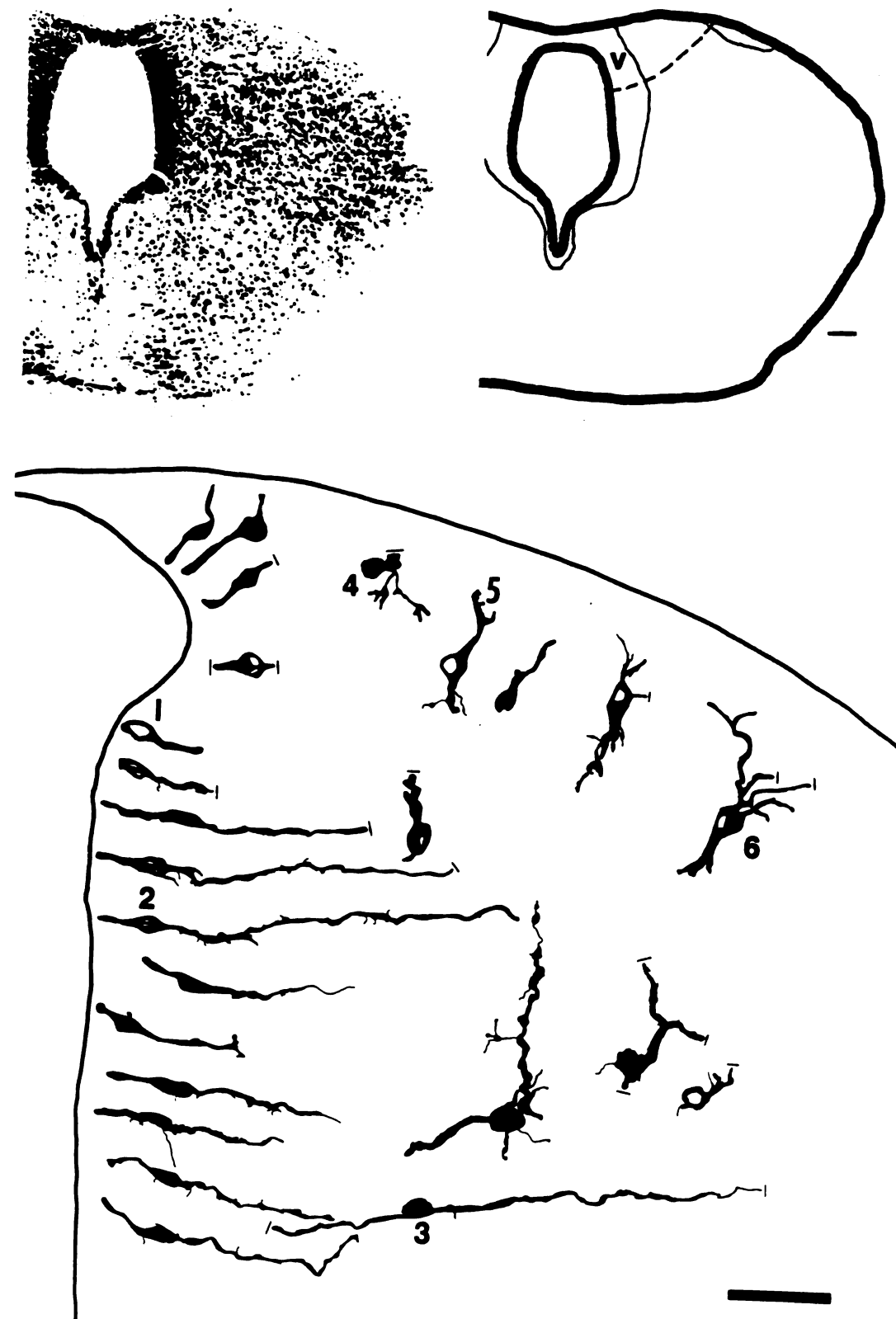

Figure 3.

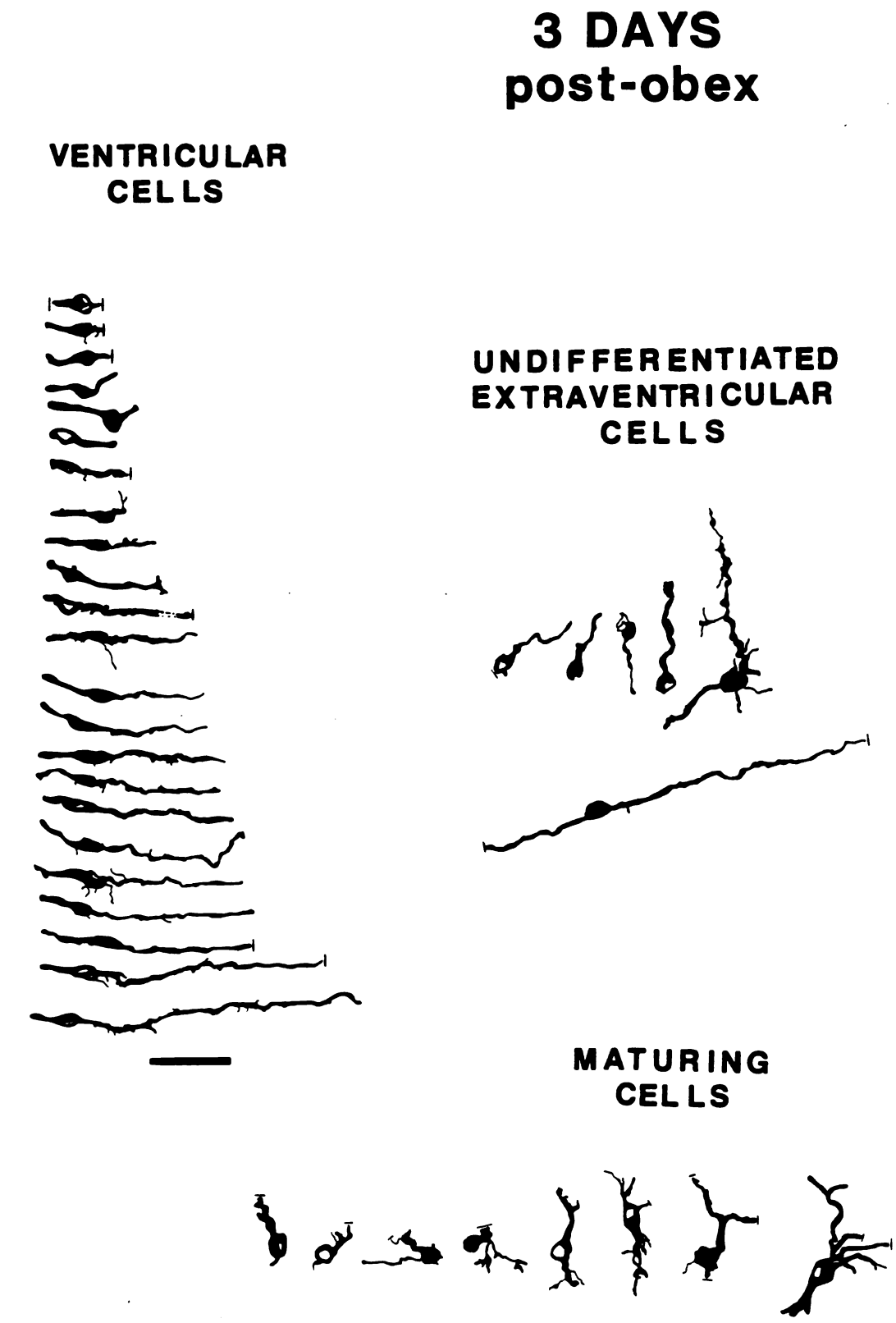

Figure 3 (cont'd)

Figure 4. Example of 3 day old, pre-obex. A sample of cells rostral to the obex from the same specimen illustrated in Figure 3. 1 - ventricular cell with no external process. 2 - ventricular cell with oval translucent patch. 3 - ventricular cell with opaque bar through translucent patch. 4 - undifferentiated extraventricular cell having little more than a soma. 5 - undifferentiated extraventricular cell with an internal radial fiber. 6 - maturing cell similar to bouquet cell in morphology. 7 - maturing cell similar to stellate in morphology. V - ventricular zone.

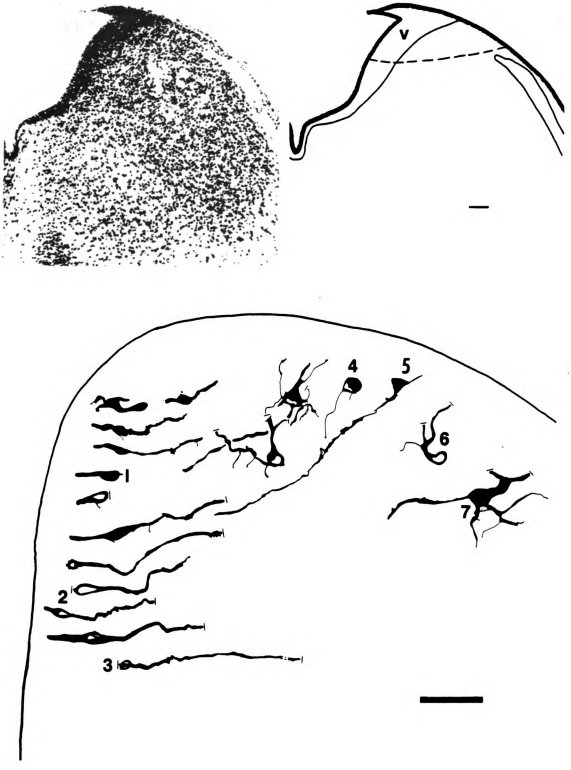

Figure 4.

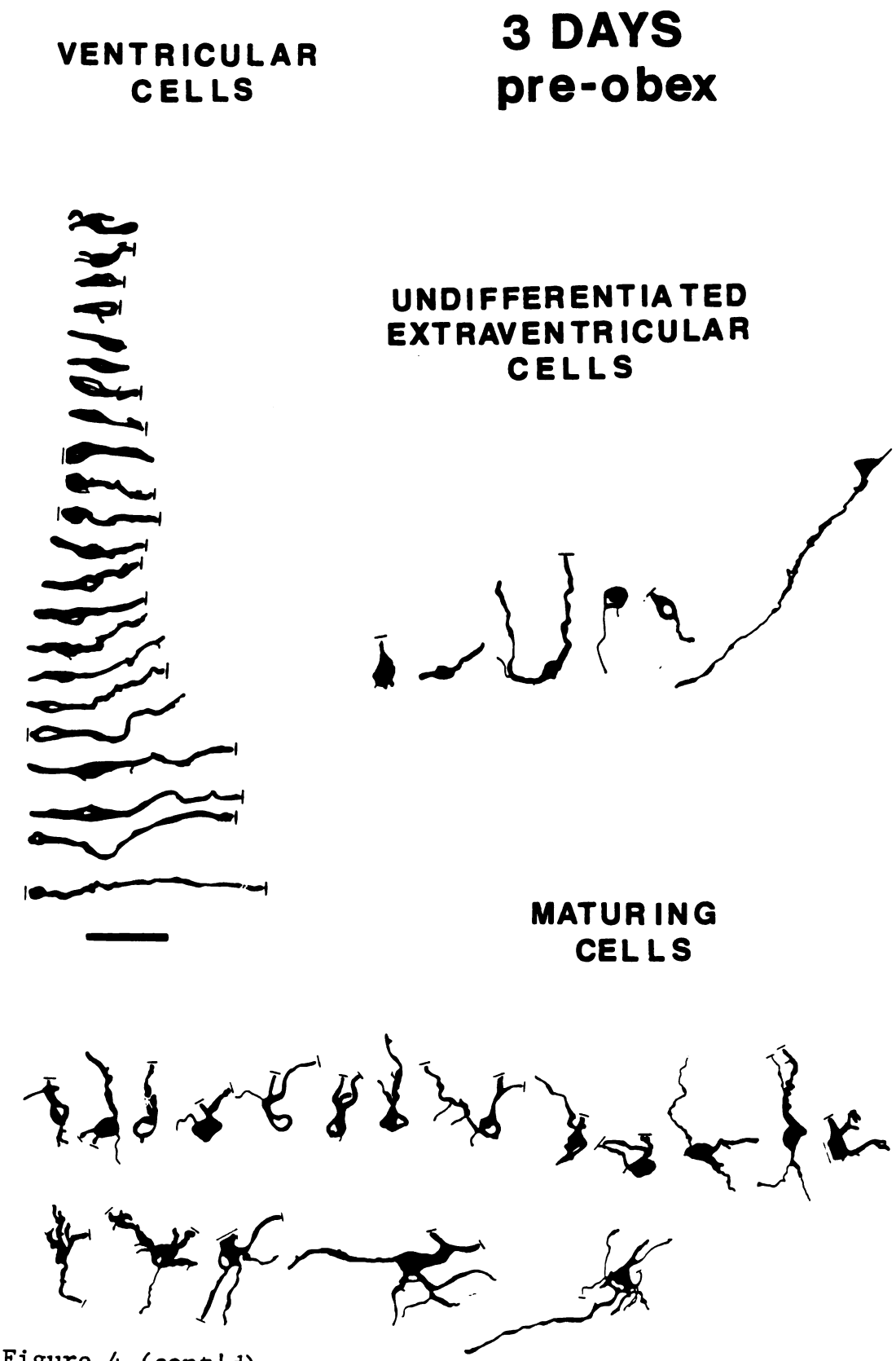

Figure 4 (cont'd)

Figure 5. Example of 5 day old, post-obex. A sample of cells caudal to the obex during the 4-6 day period. 1 - dorsal median ventricular. 2 - undifferentiated extraventricular cell which has migrated laterally from the dorsal median region. 3 - ventricular cell with a translucent soma. 4 - ventricular cell with an opaque bar through a translucent patch. 5 - maturing cell similar to bouquet cell in morphology. 6 - maturing cell similar to stellate cell in morphology. Arrows point to sheet-like outgrowths. V - ventricular zone, g- gracile, c- cuneate.

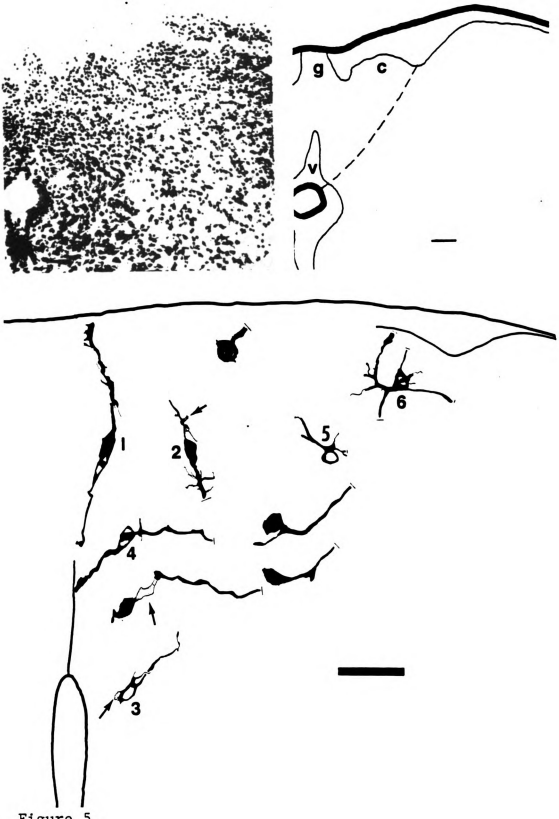

Figure 5.

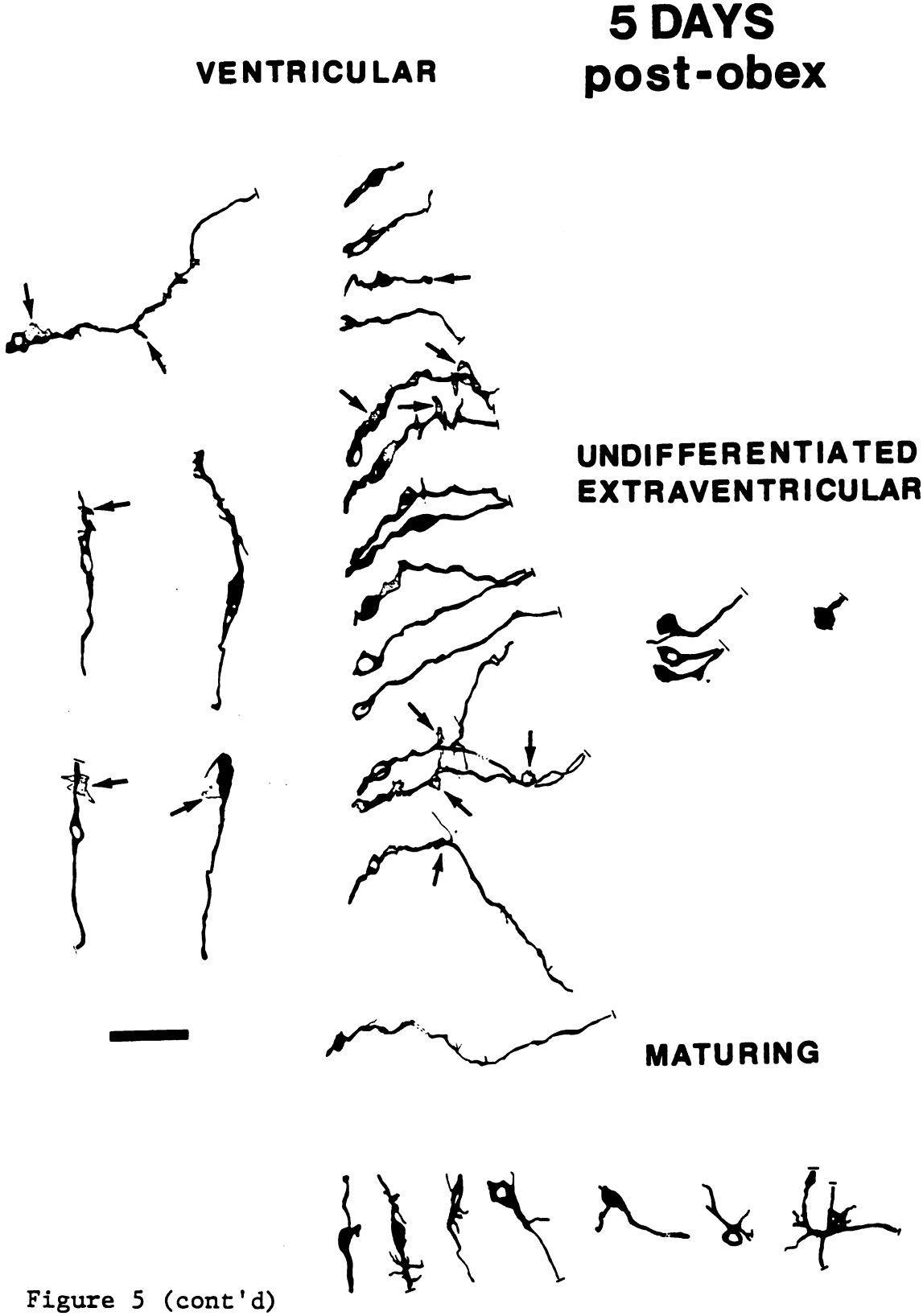

Figure 5 (cont'd)

Figure 6. Example of 6 day old, pre-obex. A sample of cells rostral to the obex during the 4-6 day period. 1 - putative contacts involving ventricular cells and radial fibers; photomicrographs of these contacts are shown in Figure 12. 2 - putative contacts by filopodia-like processes (arrows) involving a ventricular cell and neighboring radial fibers. 3 - undifferentiated extraventricular cell having little more than a soma; it appears to contact the nearby radial fiber. 4 - maturing cell within the ventricular zone. 5 - descending axon which may interact with a radial fiber. 6 - undifferentiated extraventricular cell with relatively smooth, branched processes and a prominent sheet-like outgrowth (arrow). V - ventricular zone, g- gracile, c- cuneate.

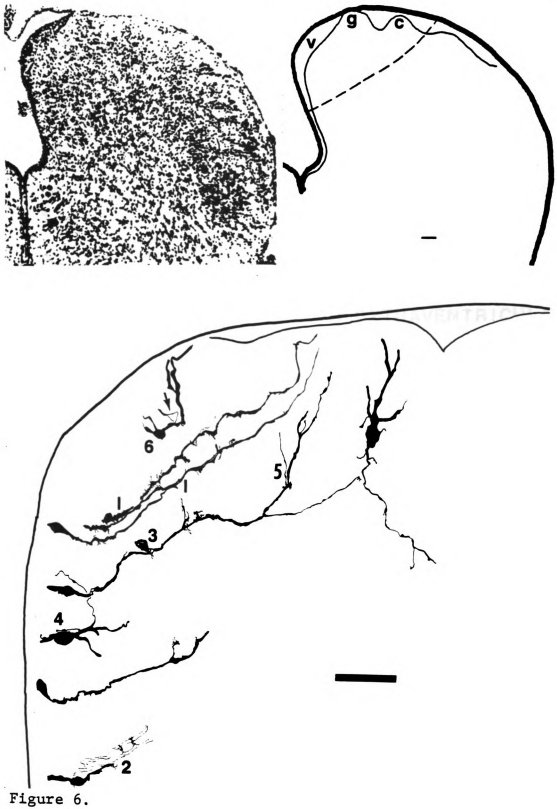

Figure 6.

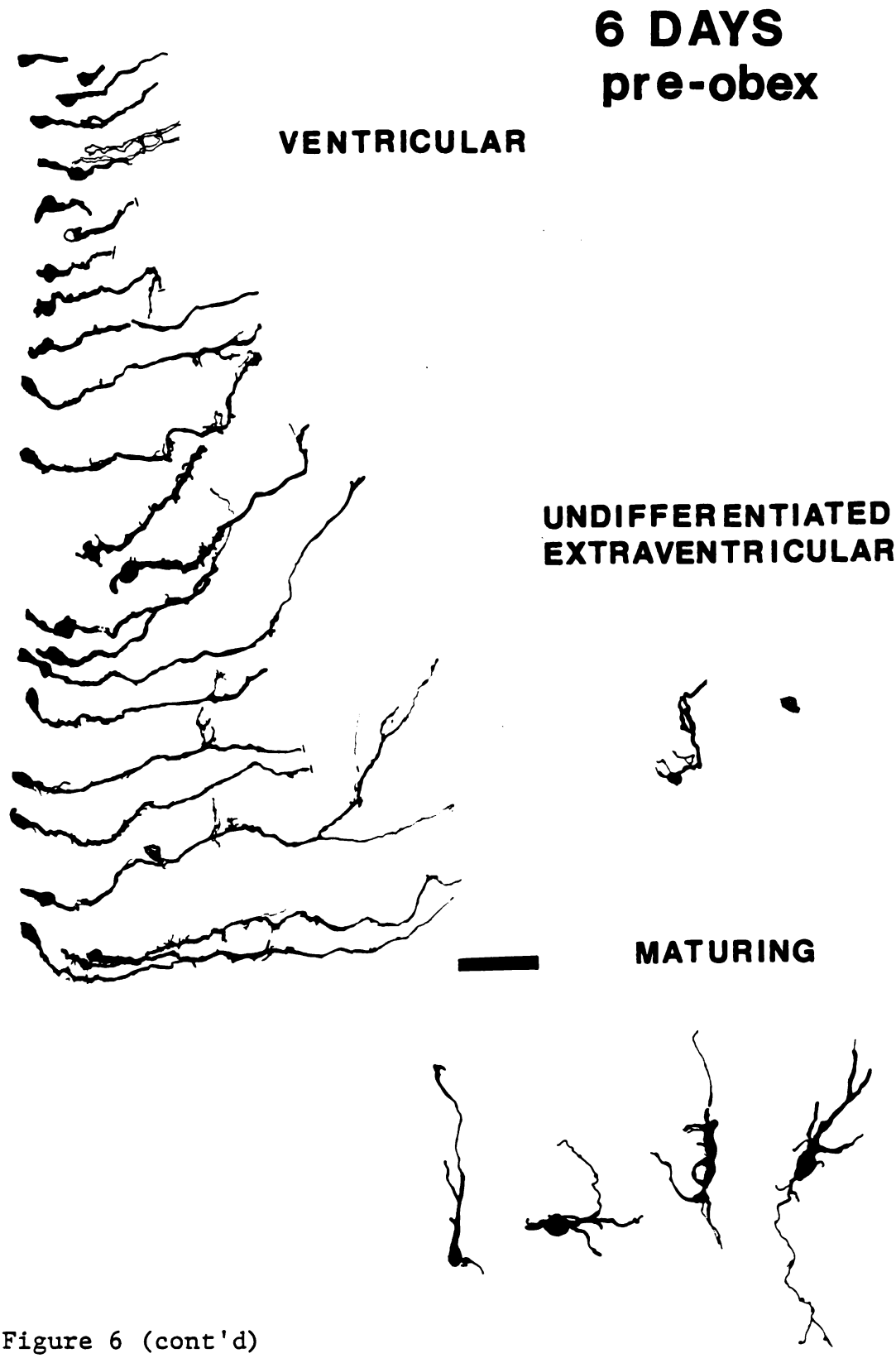

Figure 6 (cont'd)

Figure 7. Example of 8 day old, post-obex. A sample of cells caudal to the obex during the 7-9 day period; Golgi-Cox impregnation. 1 - undifferentiated extraventricular cells. 2 - maturing cells. Ventricular and undifferentiated extraventricular cells from the dorsal median region are illustrated on the right hand page (Bar equals 10 μm). Photos from four different planes of focus are shown. Notice the thicket of cell bodies (letters) and radial fibers (arrows). Some putative contacts are identified by arrow heads. V- ventricular zone, g- gracile, c- cuneate (in drawing only).

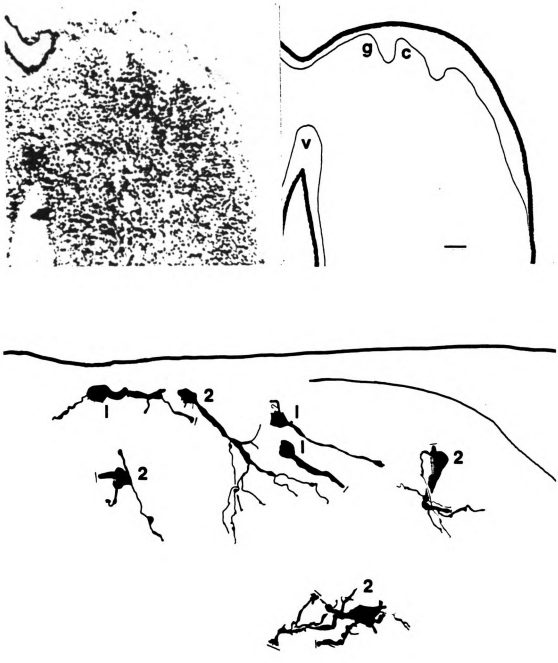

Figure 7.

Figure 7 (cont'd)

Figure 8. Example of 12 day old, post-obex. A sample of cells caudal to the obex during the 10-12 day period. 1 - ventricular cell with a translucent oval patch. 2 - undifferentiated extraventricular cell with little more than a soma present. 3 - maturing cell in the ventricular zone. Arrows point to sheet-like outgrowths. V- ventricular zone, g- gracile, c- cuneate, t- tear in the tissue.

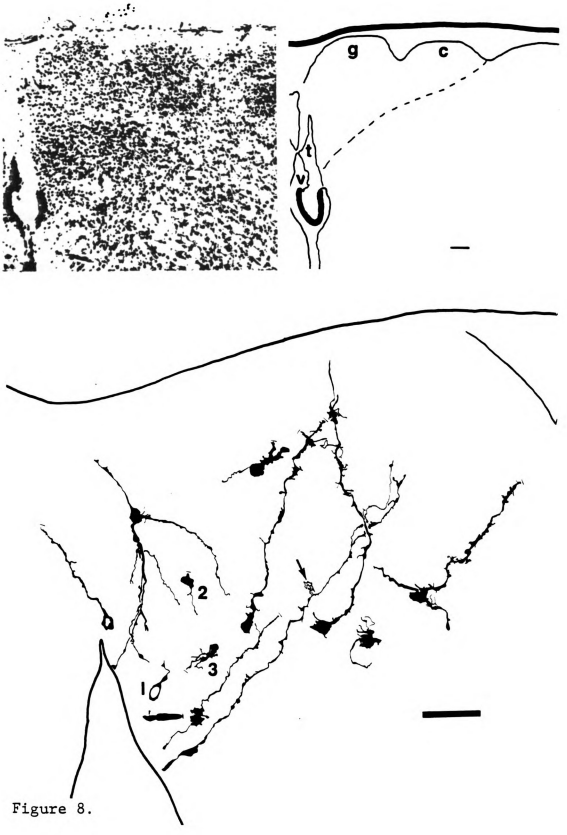

Figure 8.

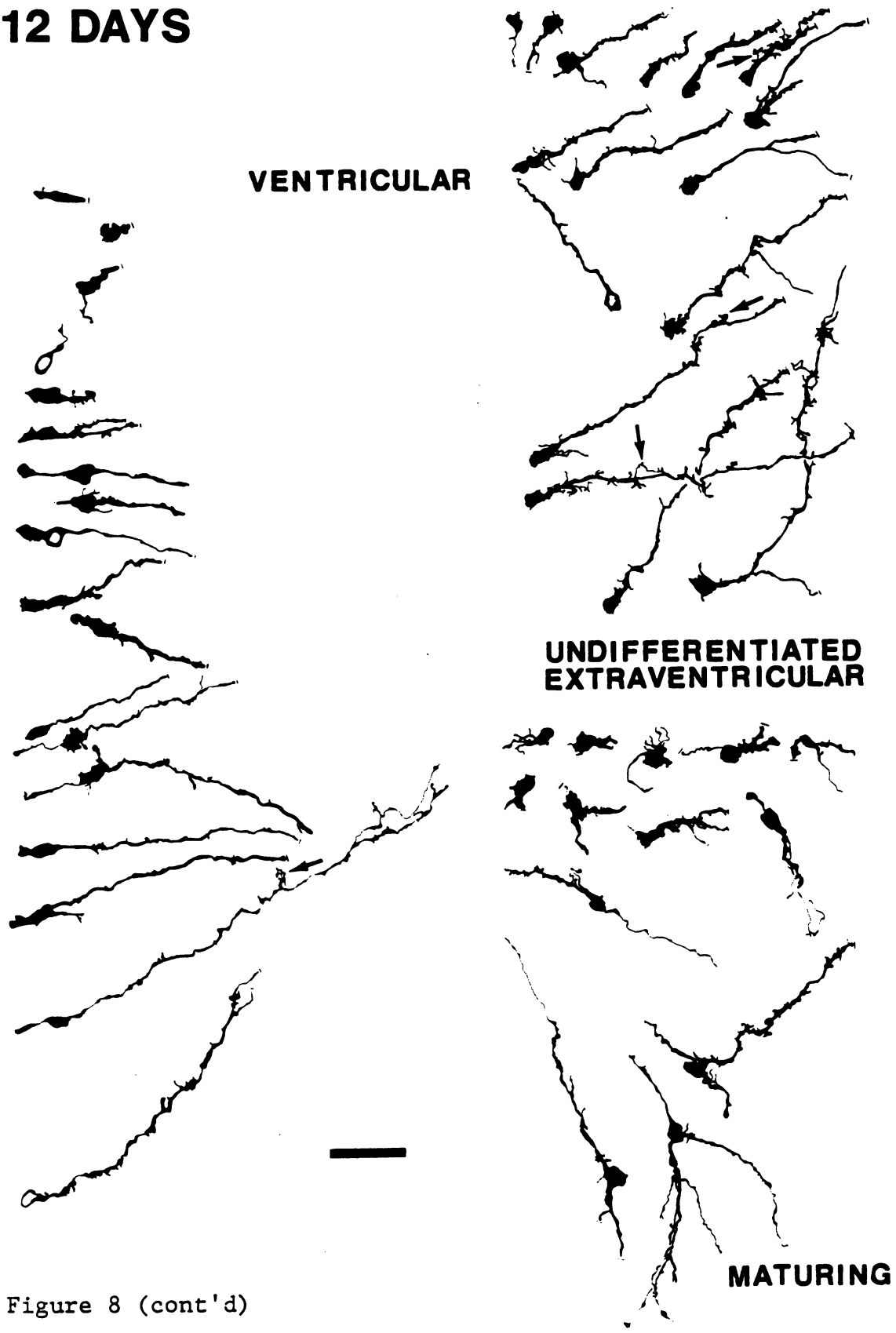

Figure 8 (cont'd)

Figure 9. Example of 18 day old, post-obex. A sample of cells caudal to the obex during the 16-18 day period. The illustrated Nissl section is from a 16 day old animal. 1 - maturing cells similar to elongate cells in morphology. 2 - maturing cell similar to bipolar cell in morphology. 3 - undifferentiated extraventricular cell with little more than a soma. V- ventricular zone, g- gracile, c- cuneate.

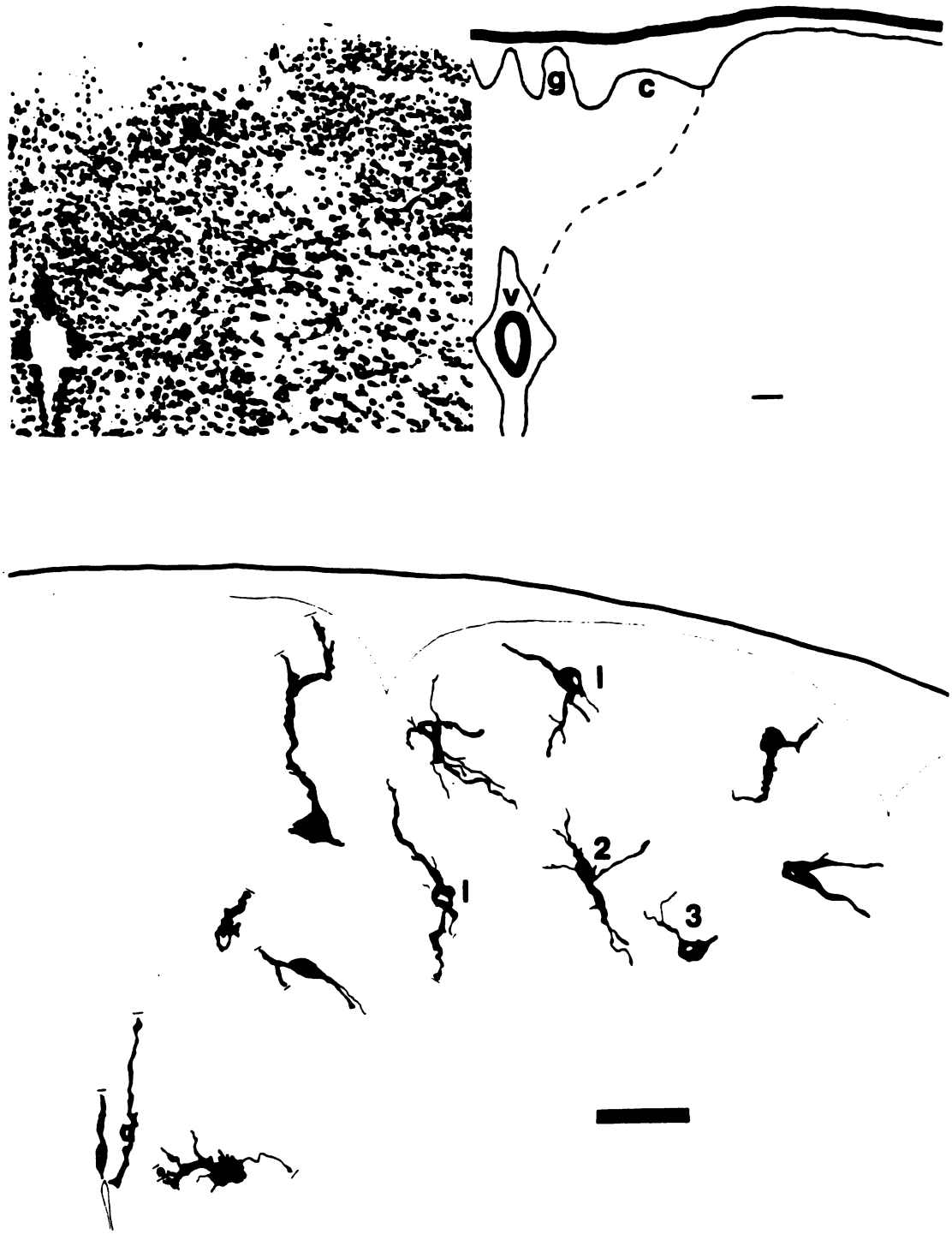

Figure 9.

18 DAYS

Figure 9 (cont'd)

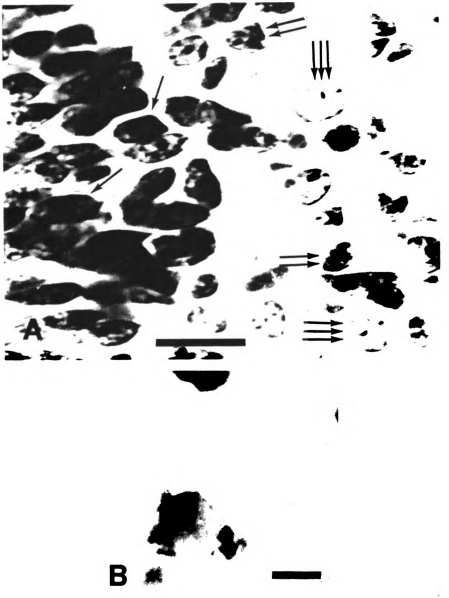

Figure 10. Nissl Cell Types. A- Three day old opposum. Cells in the ventricular zone are to the left. Single arrow - ventricular cell: deeply basophilic, bipolar ovoid in the ventricular zone. Double arrow - undifferentiated extraventricular cell: same staining characteristics as ventricular cell but lies outside the ventricular zone. Triple arrow - maturing cell: appears as a round, clear nucleus containing clumps of chromatin and little or no staining in the perikaryon. B - adult Nissl stained cell. Bar equals 10μm.

Radial Fibers. At low magnification one sees a broad sheet of fibers radiating from all parts of the ventricule toward the pial surface (Figure 11A). Upon closer inspection quite a bit of variation in these radial fibers is apparent. The most common radial fiber is the external process of the V and UXV cells. The direction of this external process is taken to be the migratory destination of the V cell. Radial fibers also emerge from UXV and M cells which are directed toward the ventricle. However, in this case the radial fiber has never been observed to end within the ventricular zone. The origin of this type of radial fiber from the cell body is occasionally off to the side rather than from the longitudinal pole (e.g., Figure 3, cell 3). A third population of radial fibers does end within the ventricular zone, usually in a bulbous ending (e.g., Figs. 11B, C). In no case could these fibers be followed to an obvious cell body outside the ventricular zone. Although the lateral extent of the process was of ten obscured in a dense precipitate or cut out of section, this was not always the case (e.g., Figure 11B). These fibers likely contain a small nucleus with an inconspicuous perikaryon.

A striking finding is what appears to be contacts between neighboring radial fibers and between radial fibers and V cell somata (Figure 12). The radial fibers involved in these contacts are often those which end in the ventricular zone but have no salient somata. The possibility of contacts involving radial fibers suggests that the opaque bars seen through a translucent V cell body and the apparent sideward

origin of some UXV radial fibers may be further evidence of contact.

Five kinds of putative contacts are seen:

(1) between asomatous radial fibers and V cell somata.

(2) between asomatous radial fibers and radial fibers of V cells.

(3) between radial fibers of V cells.

(4) between radial fibers and UXV somata.

(5) by means of protoplasmic bridges between radial fibers.

Birth to 3 days (Figs. 3, 4). By the beginning of this period the obex neural tube has just closed or is about to close. A thin strip of the ventricular zone lies between the dorsal median pia and the ventricle. Near the dorsolateral surface M cells are quite common. There is a strong tendency for the orientation of the M cells to be normal to the pia. The V cells are consistently mediolateral in orientation with little curvature to their external processes. There are no apparent differences between pre- and post-obex cells.

Day 4 to 6 (Figs. 5, 6). During this period the characteristic dorsal outline of Cu-Gr appears as the dorsal columns form. Axons are observed descending into the region and may begin interacting with cells (e.g., Figure 6). Sheet-like outgrowths are common during this period.

Differences between pre- and post-obex regions are evident. Caudally, V cells are now present along the dorsal

midline. Rostrally, the external processes of V must be longer than their caudal counterparts in order to reach the dorsal surface. They are more roughly varicose and tend to have a greater curvature.

During this period we first see the apparent contacts involving radial fibers. In a 6 day pre-obex specimen one sees what may be another form of interaction between V cells and radial fibers: wispy filopodia-like processes from one radial fiber intertwine with another (Figure 6).

Day 7 to 9 (Figure 7). It is known that during this period synapses first appear and attain their adult density (Tweedle et al., 1977). We now see a sharp decline in the relative number of V cells (see Appendix 1). This is observed in both Golgi and Nissl material. Dorsoventral V cells along the midline are still present, but mediolaterally oriented V cells are no longer found. Instead the more lateral V cells are obliquely oriented.

The orientation of M cells during this period is not as predominantly normal to the pia as it was at earlier ages. Possible radial fiber contacts in the dorsal median zone of an 8 day specimen in Figure 11B.

Axon-like fibers are first seen crossing the midline in the ventral portion of Cu-Gr.

From this age on one sees roughly contoured somata of many lateral V and UXV cells.

Day 10 to 12 (Figure 8). The rostro-caudal difference in radial fibers which was mentioned during 4-6 days is no longer apparent. Perhaps due to the increased distance from

the ventricle to the pia, the external processes of the post-obex cells now look similar to those of pre-obex cells.

The decussating axons ventral to Cu-Gr are quite common. Descending dorsal column axons can be observed giving off a number of collaterals.

Day 13 to 15. This period is not very different from the previous one. We still see V, UXV, and M cells.

Day 16 to 18 (Figure 9). Dorsolateral V cells are no longer seen from this age on. However, dorsal median V cells are still present, as well as UXV and M cells.

Thin tortuous axons are seen with adult-like bulbous endings. However, the plexi do not attain the density of dorsal column terminals seen in the adult.

Figure 11. Radial fibers. A. Fibers radiating between the ventricle and pial surface in this 6 day old pre-obex specimen. The portion in the box is illustrated in Figure 6. B. Asomatous radial fiber from an 8 day old, Golgi-Cox impregnation. A small bulbous ending lies in the ventricular zone. No perikaryon is clearly apparent. C, D. Two planes of focus through the same field from a five day old specimen. Double headed arrow points to radial fibers with bulbous endings in the ventricular zone. The lateral termination of these processes could not be followed. Arrowhead points to a possible contact between one of these fibers and a ventricular cell. A sheet-like outgrowth is also present (so). V- ventricle, d- dorsal, l- lateral. Bars equal 20 μm.

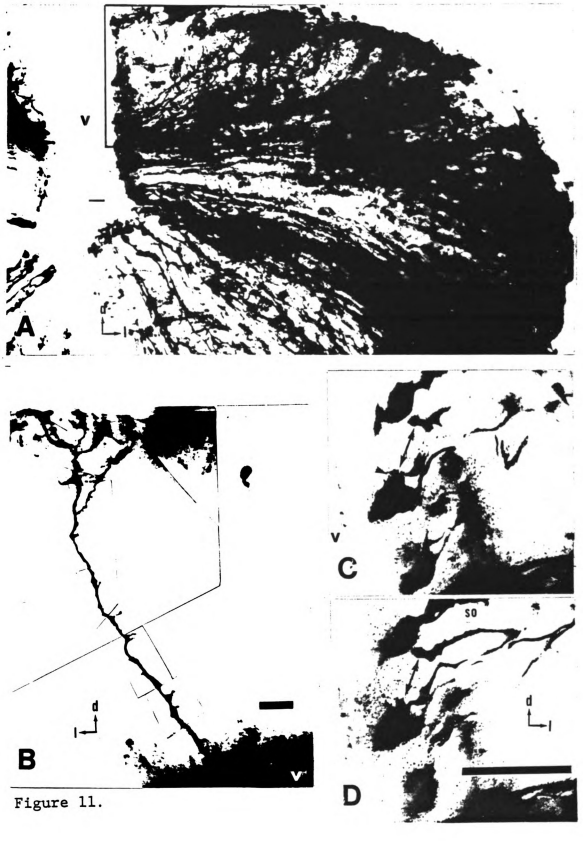

Figure 11.

Figure 12. Radial Fiber Contacts. A. Putative contacts (arrows) between a ventricular cell and a radial fiber from a 4 day old animal. B. Drawing of A. C, D, E. Putative contacts (arrows) between radial fibers and ventricular cells in a 6 day old animal (cf., Figure 6). D and E are two planes of focus of the same field. Protoplasmic bridges seem to extend themselves between the radial fibers. d- dorsal, l- lateral. Bar equals 10 μm.

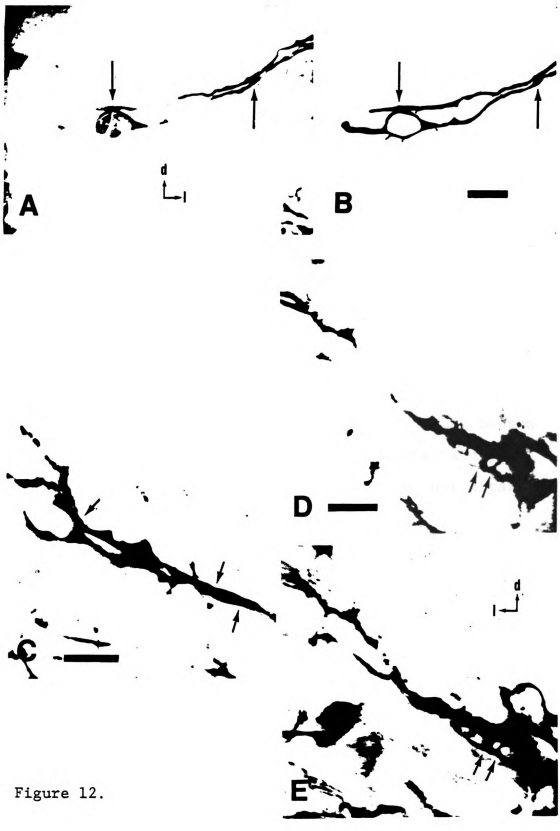

Figure 12.

DISCUSSION

The purpose of this morphological study has been to provide data on normal Cu-Gr development per se and to offer recommendations regarding the direction of research into the mechanisms underlying the central effects of early limb removal.

The most important finding has been that during the first 18 postnatal days development proceeds gradually. Furthermore, discrete phases of cell birth, migration, and differentiation are not present during this time. From the initial three-day postnatal period UXV and M cells are present in the nuclear region, but by 18 days the picture has not dramatically changed. While many M cells appear to be young neurons of a certain morphological type, adult neurons are clearly not present; the differentiation of the young neuron is at a plateau. Dendrites and dendritic branches are present in cells by 18 days, but these processes do not attain their full length until sometime after 18 days.

The axons of M cells did not discernably impregnate. Even in sections in which long projecting axons of dorsal root ganglion and ventral horn cells can be followed, axons of young Cu-Gr cells or bands of internal arcuate fibers were not detected. Therefore, while the absence of impregnated M cell axons does not necessarily imply that they are not present, their absence is probably due to some intrinsic feature of the cells and not a technical quirk. In addition, the contention that M cells do not have axons is not contradicted by the available electron microscopic evidence (Ostapoff,

personal communication, manuscript in preparation; Ostapoff, 1980). Very small EM samples of approximately 500-1000 μm^2 were examined from 9 specimens ranging in age from 3-20 days. The samples are admittedly quite restricted (total area approximately 144mm^2) and from single 60nm sections, but, nonetheless, no axon hillock was seen emerging from the cell bodies observed. One is led to the suspicion that the complete dendritic differentiation of Cu-Gr neurons may follow the establishment of their efferent connections. This phenomenon has long been known for spinal motoneurons (Barron, 1943) and, indeed, is a textbook principle of developmental neurobiology (Jacobsen, 1978, p. 187). Perhaps the plateau of M cell differentiation ends after Cu-Gr efferents would determine whether these rostral connections are indeed made after postnatal day 18.

An equally attractive, though not contradictory, hypothesis is that the full differentiation of neurons is dependent upon the activity of specialized tactile receptors, which are not present in the glabrous forepaw skin until after 18 days (Brenowitz et al., 1980). To test this hypothesis one would need to examine adult Golgi or EM material of animals which have been amputated after the close of the period during which lesions result in volume loss but before the appearance of receptors (between 15-20 days).

Golgi studies have provided much data regarding the mode of neuroblast migration. However, a uniform account of neuroblast migration does not emerge from the literature (Berry & Rogers, 1965; Stensaas, 1967; Morest, 1970; Rakic, 1971,

1972; Puelles & Bendala, 1978). The fundamental dichotomy is whether migration consists of a nuclear translocation analogous to that occurring during the mitotic cycle or occurs by a free, ameboid-like migration. Morest (1970) described the former in the opossum forebrain, while Rakic (1971, 1972) has proposed that radial glial processes assist in the free migration of neuroblasts in both cerebral and cerebellar cortices of primates. Puelles and Bendala (1978) have suggested that both types of migration are accounted for by two types of chick optic tectum neuroblasts.

Any complete description of migration must account for nondifferential migration as well as differential migration. Nondifferential migration is the passive displacement of a cell relative to a reference point, such as the ventricle. For example, a cell may move further from the ventricle solely by the addition of newborn cells along the ventricle or by the intervention of other migrating cells. We make this point because passive vectors in migration have been largely ignored in the literature and they must certainly quality in Golgi interpretations about free migration.

The evidence in this study points to two patterns of migration. Dorsal median V cells appear to follow a to and fro migration similar to the classical descriptions of germinal cell mitosis (Sauer, 1935; Fujita, 1964). Post-mitotic cells lose their attachment to the ventricle and pia and are displaced laterally (Figs. 5, 13A). The lateral migration of the cells is probably due largely to nondifferential migration, since throughout and for many weeks following the 18

day postnatal period cells are apparently being born at the dorsal midline. Ameboid-like free differential migration may have a role as well.

The more lateral V cells grow curved radial processes which reach the pia, often with a number of fine branches. No cell body outside the ventricular zone possessing such a radial fiber has been seen with an internal process still attached to the ventricle. It is therefore unlikely that the nuclei of mitotic cells from this lateral portion of the ventricular zone pass between the pial and ventricular surfaces during the different mitotic phases. Movement during mitosis is probably restricted to the ventricular zone. The migration of post-mitotic cells begins as a nuclear translocation away from the ventricle. The cell generally loses its internal process as it leaves the ventricular zone. The migratory scheme proposed by Morest for the opossum forebrain (1969, 1970) fits well with our observations of these lateral V cells in the opossum Cu-Gr (Figure 13B). The cell body appears to migrate as a nuclear translocation through the cylinder of the external process, losing its attachment to the ventricular wall as it leaves the germinal zone. Upon reaching its destination it loses its connection with the pial surface and begins to differentiate. Passive migration relative to the dorsal pial surface probably plays a minor role.

The putative contacts involving radial fibers bring to mind the demonatration by Rakic (Rakic, 1971, 1972) that radial fibers contact migrating neuroblasts in the cerebral and

Figure 13. Two proposed schemes of neuroblast migration. A. Dorsal median ventricular cells are displaced laterally, and reach their adult position largely by a passive, or non-differential migration. B. Ventricular cells from the lateral portion of the ventricular zone. This cell migrates by a nuclear translocation through the cylinder of the external radial fiber. No adult cell type is implied to be characteristic of each migratory method.

cerebellar cortices. Our data are insufficient to decide whether the contacts we see have an important role in migration. Seymour, Kemp, and Berry (1972, their Figure 15C) observed with scanning EM of germinal cells protoplasmic bridges similar to the contacts we have seen. Such contacts could conceivably represent specialized junctions through which ionic current or small molecules flow.

The two patterns of migrations suggest by their respective positions a difference between the cuneate and gracile portions of the nuclear region. In favor of this argument is the correlation that the time course of the gross development of the hindlimb trails that of the forelimb (Mizell, 1968) while dorsal median V cells begin later than and are present after the lateral V cells are no longer present. Unless the dichotomous pattern has some functional implication (it may merely reflect the distance and direction of migration), we would be quite surprised if this phenomenon represented a truly important difference between cuneate and gracile, since physiologically and anatomically these are not separate nuclei but the lateral and medial components of the same nuclear mass (cf., Johnson, 1980 for a full discussion of this point).

Glial cells cannot be distinguished in the material in question. This is not to imply that cells destined to be glia have not been born during this period. As Schmechel and Rakic (1979) point out both glia and neurons begin as bipolar ovoids with radial processes. Until sufficient differntiation has taken place they are indistinguishable. Electron

microscopic evidence suggests that glial-like processes (profiles containing microfilaments) are present by postnatal day 5 (Tweedle et al., 1977). However, during the first 20 days glial processes do not interdigitate in the interstices of other processes and somata, synapses are not insulated by glia, and myelinated fibers are not present. As a general rule glia develop after neurons (cf., Jacobsen, 1978, p. 58). The dorsal median V cells may therefore be a major source of Cu-Gr glia. They do not appear until many M cells are present in the nuclear region, and they are still apparent as late as 90 days (snout-rump length 185 mm) after birth.

On the effects of early limb removal: reevaluation and recommendations. Three possible changes could account for the volume loss in Cu-Gr following peripheral damage: (1) cell death due to transsymaptic degeneration or the enhancement of normal cell death, (2) an attenuation of cell volume due to growth retardation or atrophy, and (3) a subnormal amount of cell birth.

Normal cell death may not be an important aspect of Cu-Gr development. Ulinski (1969) concluded as much based on Nissl cell counts. Allowing for the fact that cell counts in this area having an indistinct ventral boundary are somewhat unreliable (see also Schreck & Johnson, 1978), Ulinski (1969) found that the number of cells in cuneate increases until sometime after postnatal day 16, after the age at which volume loss following forepaw removal has first been seen (i.e., day 15, Schreck & Johnson, 1978). Dying cells in

normal preparations were not seen in that Nissl study or in EM or 1 μm methylene blue stained sections (Ostapoff, personal communication, manuscript in preparation; Ostapoff, 1980). Neither has cellular degeneration been observed 3, 6, 9, or 11 days following limb removal (Ostapoff, 1980). If degeneration is occurring it might be happening at a very slow rate, commensurate with the rate of nuclear development. A slow rate of degeneration would explain why degenerating profiles are not seen. If, for example, only 20 cells in the entire nuclear region degenerate per hour following hand removal, their detection in fixed tissue (a snapshot of the developmental picture) would be unlikely, especially in EM samples. But from the lesion at day 9 to the observed volume loss 6 days later 2880 cells would have died on the lesioned side, approximately 25% of the cells normally present in cuneate (Ulinski, 1969). A search for degenerating cells in 6 hour intervals following hand removal is now underway. If no degeneration is found in those cases it is time to turn our attention to the other hypotheses.

Atrophy or growth retardation may occur in the Cu-Gr ipsilateral to a limb removal. Relative volumes of EM profiles, however, do not differ between experimental and control sides of lesioned animals (Ostapoff, 1980), as would have been expected if atrophy or growth retardation had occurred. Golgi impregnations of amputated animals might also shed some light on this hypothesis.

The most readily testable hypothesis and the one most strongly suggested by the present data is the third: Cu-Gr

volume loss due to limb removal is due to a subnormal amount of cell proliferation. Lateral V cells, the cells most likely to end up in cuneate, are not seen after 15 days. Hand removal at 15 days does not result in a loss of cuneate volume (Schreck & Johnson, 1978). Does the end of susceptibility to injury correspond to the end of lateral V cell germination? If so, volume loss may be due to a subnormal amount of cell birth. This hypothesis implies that afferent imput plays a role in controlling cell proliferation. Studies in the developing visual system argue against the notion that deafferentation has an effect on cellular mitosis (Cowan et al., 19681 Currie & Cowan, 1974). However, the coincidence we observe of synaptogenesis with a relative decrease of cells in the ventricular zone (Appendix 1) also hints at a causal relationship between afferent axons and cell proliferation. Tritiated thymidine experiments are clearly called for to resolve these issues.

This study has raised a number of hypotheses and unanswered questions which tritiated thymidine experiments can critically address: What is the time course and rate of cell birth? By what age are all Cu-Gr neurons born? At what age are glial cells first born? Is there a sharp decline in cell birth at 7-9 days? Does cuneate cell birth end between 9 and 15 days? Are the two proposed migration schemes correct? Does removing a limb result in a decrease in cell proliferation on the injured side?

Light and electron microscopic searches for afferent and neuronal degeneration at each 6 hour interval following hand

removal are now in progress for the 48 hours following forepaw removal. Perhaps these EM studies would benefit from closer attention to tissue in and around the ventricular zone, where V and UXV cells are common, since this study raises the suspicion that they may be important in mediating volume loss.

These two experimental approaches, studies of cell birth and degeneration, hold the most promise for providing important pieces of the volume loss puzzle. Even if they fail to provide the definitive answer, they will point us down a more certain path.

Two other studies on the normal development of the opossum Cu-Gr should follow this one. An electron microscopic investigation of the ventricular and subventricular zones would illuminate the nature of the putative contacts we have seen involving radial fibers. Could they be specialized junctions?

An HRP determination of the onset of Cu-Gr efferent projections would tell us when the M cells have made their rostral connections and perhaps point to the importance of efferent contacts in the maintenance of nuclear volume following amputations after 15 days.

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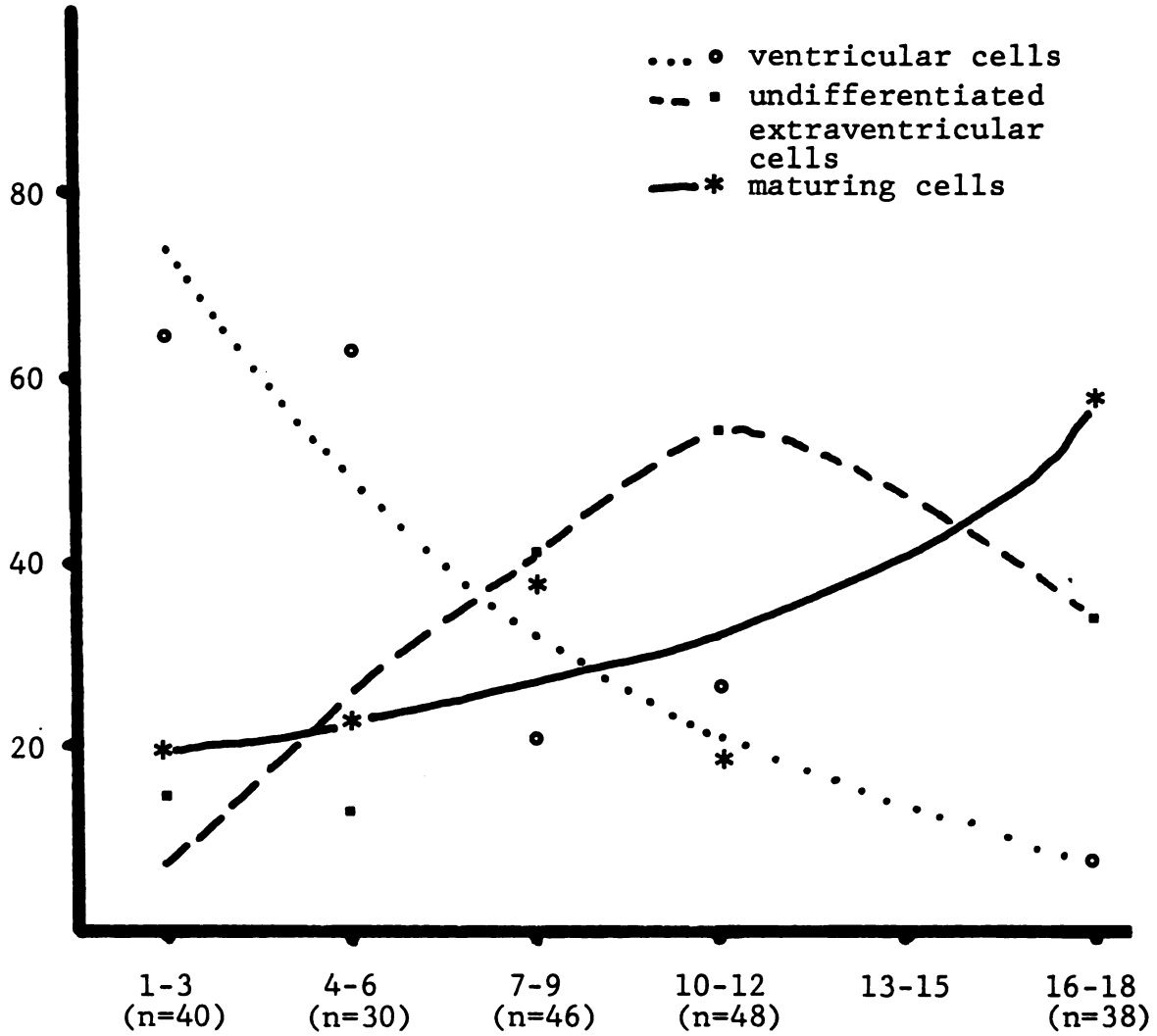
APPENDIX A



Appendix A. Time-course of developmental all types. Postnatal periods of 3 days are along the abscissa. Percent of cell types categorized caudal to the obex during each period are represented by the ordinate.