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REVIEW

The Discovery of the Matrix Cell, the Identification of the Multipotent Neural Stem Cell and the Development of the Central Nervous System

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ABSTRACT. **In the early 1960s I applied ^{3}H-thymidine autoradiography to the study of the cells constituting the neural tube, and found that its wall was composed solely of one kind of single-layered epithelial cell, which perform an elevator movement between the mitotic and DNA-synthetic zones in the wall in accord with the cell cycle. They were identified as multipotent stem cells of the central nervous sytem (CNS) to which I gave the name of *matrix cells*. ^{3}H-thymidine autoradiography also revealed the chronology of development of these matrix cells: At first they proliferate only to expand the population (stage I), then switch to differentiate specific neuroblasts in given sequences (stage II), and finally change themselves into ependymoglioblasts, common progenitors of ependymal cells and neuroglia (stage III). Based on these findings, I proposed a monophyletic view of cytogenesis of the central nervous sytem. This matrix cell theory claiming the existence of multipotent stem cells has long been the target of severe criticism and not been accepted among neuro-embryologists for a long time. Recent findings by experimental and clinical neuroscientists on the importance of stem cells have renewed interest in the nature and biology of the multipotent neural stem cells. The present paper describes how the concept of the matrix cell (multipotent neural stem cells *in vivo*) emerged and what has come out from this view over the last 45 years, and how the basic concept of the matrix cell theory has recently been reconfirmed after a long period of controversy and neglect.**

Key words: matrix cell/neural stem cell/CNS/development/^{3}H-thymidine autoradiography/monophyletic theory

1. Historical background

When Wilhelm His (1889) studied the histology of the neural tube, he was deeply impressed by the resemblance in cellular composition between the neural tube and the seminiferous tubule of the testis. Germinal cells (Keimzellen) in the seminiferous tubule, which produce spermatocytes, were rounded and located directly beneath the outer surface of the tubule frequently showing mitotic chromosomes. In the neural tube, large rounded cells were located immediately beneath the luminal surface and always showed mitotic figures (A in Fig. 1). He was convinced that both of them were "germinal cells" producing the parenchymal cells in both tissues, spermatocytes in the testis and neurons in the neural tube. Other than these cells, in both tissues there were elongated cells showing no mitotic figures: Sertoli cells in the testis and the spongioblasts in the neural tube (B in Fig. 1). He inferred that they were supporting (stromal) cells. So His formulated the basic concept of neuro-embryology, which predominated most of the 20th century: that "the neural tube was composed of two kinds of committed progenitor cells, neuroblast-producing germinal cells and glial precursors called spongioblasts". According to His' dualistic theory, all the neurons are produced from actively proliferating germinal cells, while the neuroglia are derived from mitotically inert spongioblasts, which are elongated bipolar cells developing into the syncytial myelospongium.

In 1920s, remarkable progress in neurosurgery of brain tumors was made by Cushing and his school, and neurosurgeons urgently needed to establish diagnosis of brain tumors based on the correct cytogenesis, in order to know the prognosis and to select the most suitable surgical treatment for the patients. Bailey and Cushing (1926) and Penfield (1932) proposed respectively new schemes of cytogenesis of the CNS, which were widely accepted among neuro-

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Abbreviations: CNS, central nervous system; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM fractograph, scanning electron-microscopic fractograph.

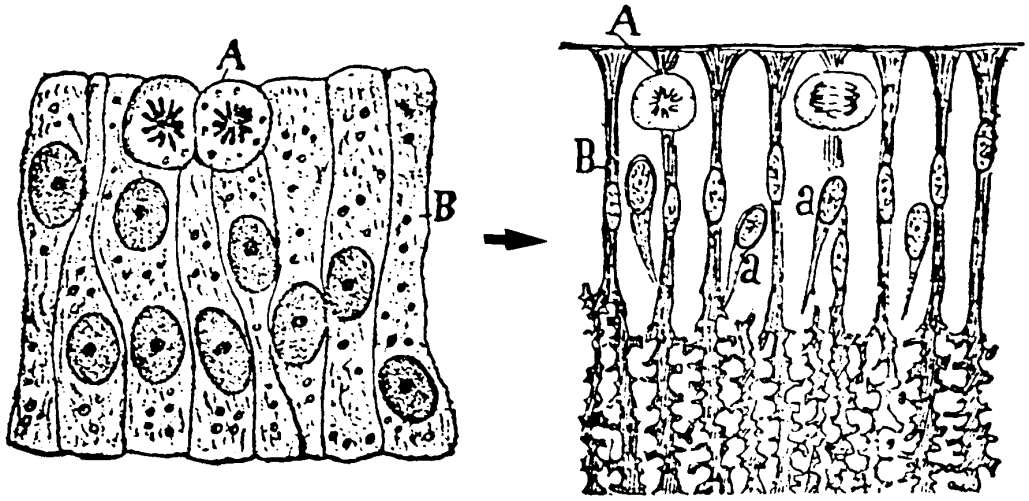


Fig. 1. Germinal cells and spongioblasts (His' own illustration, 1889). Germinal cells (**A**) and spongioblasts (**B**). Left: Wall of neural tube at the time of neural tube closure. Right: A slightly more developed neural tube. **a** indicates neuroblast produced from the germinal cells (**A**).

embryologists. These schemes, although slightly different from each other, equally adopted the most important principles of His' theory. Thereafter, the spongioblasts were sometimes called by different names of "subventricular cells" or "radial glia", and the germinal cells, by name of "ventricular cells", but the basic concept of His' dualistic theory was kept unchanged among the neuro-embryologists (cf. Levitt *et al.*, 1981).

When I began to study the cellular composition of the neural tube in 1958, the basic concept that "There should be no multipotent stem cells capable to differentiate into both neurons and glial cells" was established as an unshakable principle in neuro-oncology and neuro-embryology.

2. Encounter with a brain tumor derived from the neural stem cell

In 1958, an encounter with an autopsy case of a curious huge primary brain tumor in a 2-year-old girl, measuring 10 cm in maximal diameter infiltrating the lateral ventricle and the basal cranium (Fig. 2A), initiated me into the fascinatig world of neuroscientific studies which I continue until the present.

Histological examination revealed that the major composition of the tumor resembled neural tubes (Fig. 2B, C) together with medulloblastoma-like structures but no sign of teratoma, thereby suggesting its origin from the most immature stem cells of the central nervous system. I diagnosed it as "medulloepithelioma" (Fujita, 1958). This histological diagnosis was not accepted at all at that time because its counterpart of an originating multipotent neural stem cell in normal histogenesis was believed absolutely non-existent by the authorities of neuro-embryology and neuro-oncology. I believed in my diagnosis, however, and sent a letter to Prof. R.A.Willis of Leeds University, who had severely criticized all the reported cases of medulloepithelioma and even rejected that entity. I enclosed all my materials necessary for the diagnosis and asked him for his opinion. Quite unexpectedly, I received a very kind answer from him with his full consent to the diagnosis, in which he wrote "This is the first authentic case of medulloepithelioma". He introduced this case with a microphotograph and citation of my paper (Fujita, 1958) in his "Pathology of the Tumours of Children" (Willis, 1962). His letter and citation encouraged me very much. I had a presentiment that a new conception of cellular development of the CNS might be buried in this case report and I thought that I should not miss this precious message from the innocent child.

3. Finding of the peculiar mitotic pattern in neural tubes of human embryos

As the presence of multipotent neural stem cells in the CNS was strongly suggested from the examination of this brain tumor as mentioned above, I decided to examine neural tubes of human embryos by myself using a microscope (Fujita, 1960, Fujita *et al.*, 1960). To my great surprise, under the microscope I noticed a very characteristic mitotic pattern in the neural tube as shown in Fig. 3. The major finding can be summarized as follows.

(1) So-called germinal cells are rounded and always lie directly beneath the luminal surface showing mitotic chromosomes (M in Fig. 3A). In this *M*-zone no interphase cell is found. (2) Between the mitotic zone (*M*) and the deep nuclear zone (*Intrph* in Fig. 3A), an intermediate zone (*P & d*) with sparse nuclei exists, where only nuclei at the latter half of telophase and early prophase are to be found.

Once I had recognized this peculiar mitotic pattern of the

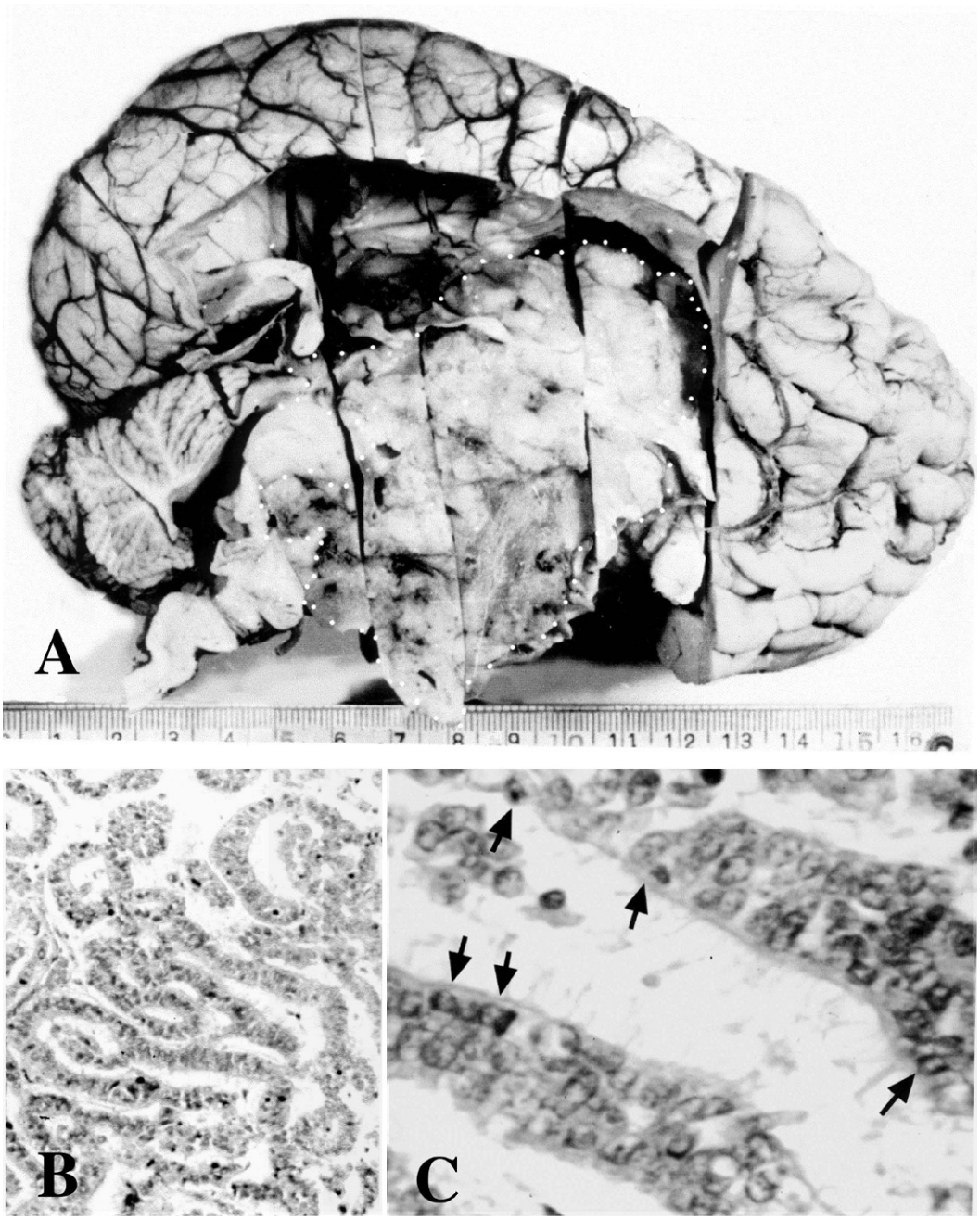


Fig. 2. Medulloepithelioma, a brain tumor originated from neuroepithelial stem cell of the CNS. (A) The left hemisphere of the brain cut on the median plain. A part of the medial wall was removed to expose the part of the tumor infiltrating into the lateral ventricle. The tumor also infiltrated into basal cranial bone (center, bottom), thereby revealing its extraordinary malignancy. The white dotted line indicates contour of the tumor. This tumor was diagnosed as "medulloepithelioma" by its histogenetic characteristics and malignant behavior. (B) Histology of the tumor (×150). Tubular structures resembling the primitve neural tube represented the most characteristic feature of the tumor. These tubular structures often merged into medulloblastomatous areas, which resembled embryonic differentiation of the mantle layer (not shown in this picture). (C) Higher magnification, ×600. The neoplastic cells composing the tubular structure showed mitotic figures directly beneath the luminal surface (arrows). Some of the neuroepithelial cells possessed faintly stained tongue-like processes, which jutted out from the apical surface of the cell into the lumen. These were the "kuppen-förmige" protoplasimc processes described by Sternberg (1927) as characteristic for the most primitive neuroepithelium of human embryo. The primitive neuroepithelial cells do not possess multiple cilia as seen in the ependymal cells but occasionally single cilium (Fujita and Fujita, 1963, 1964; Tramontin *et al.*, 2003). Adapted from Fujita, 1958.

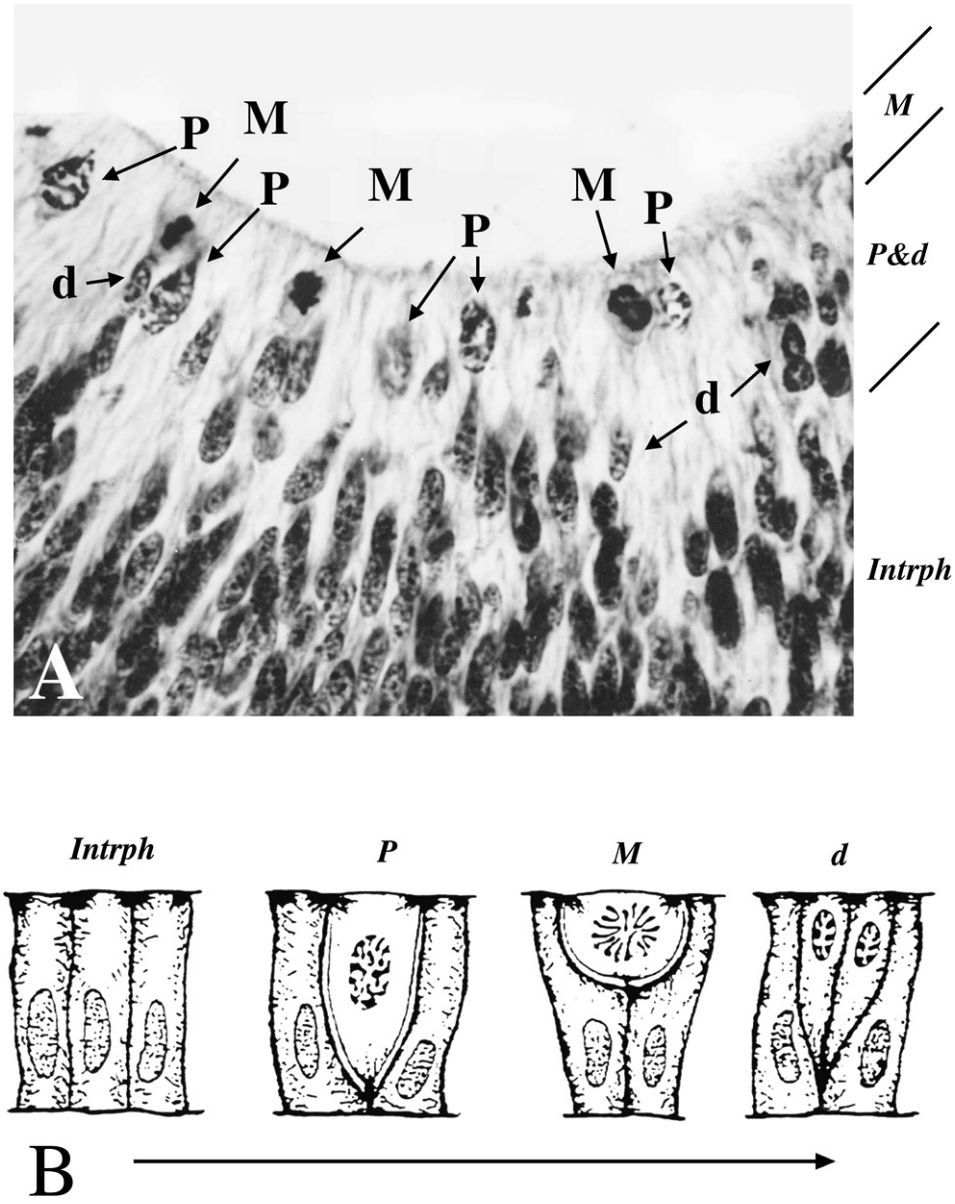


Fig. 3. Mitotic pattern in the neural tube. (A) The wall of the neural tube of the human embryo (CR length 12 mm). *M*, mitotic zone; *P & d*, intermediate zone; *Intrph*, deep nuclear zone in which interphase nuclei (of so-called spongioblasts) are packed densely (×500); d, nucleus of daughter cell; M, mitotic figure (so-called germinal cell); P, prophase nucleus. Adapted from Fujita, 1960. (B) Mechanism of the to-and-fro movement is speculated to be due to cyclic changes of cell adhesion to neighboring cells. Adapted from Fujita *et al.* (1960). When the interphase cell (*Intrph*) enters into mitosis, intercellular adhesion begins to be lost and the prophase cell (*P*) starts to round off. At the metaphase (*M*), cell membrane of the mitotic cell is completely released from the neighboring structures except for terminal bars, the cell are inevitably rounded off and attracted toward the luminal surface. After division, two daughter cells (*d*) recover the intercellular adhesions, extend their cytoplasmic processes and resume downward migration.

neuroepithelium, the wide distribution of the same pattern among proliferating tissues soon attracted my attention. I found this same mitotic pattern as described in (1) and (2) above in the following tissues: Adult: the epithelium of the efferent duct of the epididymis; the gastric mucosa; the intestinal mucosa; the epithelium of the endometrial gland. Embryo: the epithelium of the branchial clefts; the tracheal and lung anlage; the gastric mucosa; the intestinal epithelium; the epithelium of the bile duct system including the gall-bladder; the epithelium of the pancreas (these six tissues are of endodermal origin); the mesonephric tubule; the mesonephric duct; the metanephric tubule; the thickened part of the mesothelium (these four are of mesodermal origin); the epithelium of the nasal pit; Rathke's cleft; the

anlange of the retina; the neural tube (these four are of ectodermal origin). In short, the same mitotic pattern is found without distinction of germ layer origin.

What is common to these tissues is the fact that they consist of a single layer of cylindrical epithelium. Though the neural tube seems to be many-layered, it can be clearly demonstrated, using Golgi's silver method, that the wall of the tube is actually a modified single-layered epithelium. Therefore, I inferred that the so-called germinal cells showing mitotic figures in these epithelial tissues including the neural tube were, in fact, not cells *sui generis* but the actual cells of the epithelium in mitotic phase (Fujita, 1960, Fujita *et al.*, 1960).

4. Elevator movement of the neuroepithelial cell

These observations (Fig. 3A) suggested that the "germinal cells" and the "spongioblasts" are nothing but different phases of just one kind of epithelial cell, which perform an elevator-like movement during proliferation (Fig. 3B). The nuclei of the cells undergo a cyclic change of position in the neuroepithelial cell layer: The nuclei of intermitotic cells lie deeply, and progressively approach the ventricular lumen during G2 and prophase. Nuclear division occurs exclusively at the luminal surface, whereupon the daughter nuclei move back again to deeper positions (Fujita, 1960, Fujita *et al.*, 1960).

Schaper (1897) and Sauer (1935) had observed the same pattern and suggested similar interpretations. However, His' dualistic theory that claimed the presence of independent neuroblast-producing germinal cells and spongioblasts in the neural tube (Fig. 1) was so dominant at the time that it was impossible for neuro-embryologists to accept this interpretation of a to-and-fro movement of the nuclei in the neural tube. The findings of Schaper and Sauer went neglected for a long time, since the conception of cell cycle had remained virtually unknown among all the biologists including embryologists and anatomists until the end of 1950s.

In 1953, Howard and Pelc proposed the existence of the cell cycle based on the data of ^{32}P-autoradiography but few accepted this idea because ^{32}P incorporation was not specific to DNA synthesis. Subsequent application of ^{3}H-thymidine autoradiography, however, definitely proved the existence of the S-phase and it was in the early 1960s when the existence of the four phases G1, S, G2 and M in the cell cycle was firmly established in cell biology (Quastler, 1963). Retrospectively speaking, I was very fortunate that I was familiar with this new concept of cell cycle in the early 1960s, and was ready to interpret the microscopic findings of the mitotic pattern in the neural tube as a sequential phenomenon of G1, S, G2 and M in the cell cycle.

Application of ^{3}H-thymidine appeared to me the best experimental method to prove the movement of the neuroepithelial cells in the cell cycle and to identify the so-called germinal cells with the spongioblasts. As ^{3}H-thymidine became available to me in 1962, I applied it to autoradiography of chick embryos (Fujita, 1962) and succeeded in visualizing the intermitotic movement of the neuroepithelial stem cells in the neural tube (Fig. 4). In neural tubes of chick embryos killed 30 minutes after injection of ^{3}H-thymidine, only cell nuclei located in the deeper zone of the wall incorporated ^{3}H-thymidine (Fig. 4A). I called it the *S*-zone (zone of DNA synthesis). None of the "germinal cells" in the *M*-zone (zone of mitosis) were labeled thereby indicating that they were not independent cells. If so, they would have repeated mitoses halving DNA and always remained the same "germinal cells" without synthesizing DNA! Cell nuclei located in the intermediate zone (*I*-zone) also did not take up the label.

In autoradiographs taken 2 to 3 hr after ^{3}H-thymidine injection, however, many of them in the *I*-zone were found labeled with ^{3}H-thymidine (Fig. 4B). It is very likely that they had ascended from the *S*-zone. By 3 hr (Fig. 4B), most mitotic cells in the *M*-zone became ^{3}H-thymidine positive and thereafter the labeled cells appeared to descend passing through the *I*-zone. Thus the labeling index of the entire matrix cell layer increased until it reached 100% in 6 to 12 hr depending on the ages of the embryos. It is beyond doubt that the neuroepithelial cells perform an up- and-down movement in the neural tube synchronous with the cell cycle (Fig. 5). I called this movement *elevator movement* (Fujita, 1963).

What is the mechanism of the elevator movement? It was speculated (Fujita, 1960; Fujita *et al.*, 1960) that "the migration of the epithelial cell to the luminal surface during mitosis is considered to be associated with the cytoplasmic changes which round off the mitotic cell, releasing them from the adjacent cells except for the terminal bars" (Fig. 3B). The molecular mechanism of this dramatic change in the cytoplasmic structures, however, has remained totally unknown until quite recently as discussed later in this article (cf. Section 11).

5. Homogeneity of the neuroepithelial cell population in the early neural tube

It was fortunate for me that I first used chick embryos as the material to spare the costs of experiment, because a single injection of even a very small amount of ^{3}H-thymidine produced an effect of continuous (or cumulative) labeling. The labeling index of the neuroepithelial cells in the earliest stage of development increased linearly and soon reached 100% (Fig. 6A, B), thereby indicating that the neural tube was composed solely of proliferating neuroepithelial cells and no other kinds of cells were intermingled with them as far as the early neural tube was concerned (Fujita, 1962, 1963). The structure and location of the epithelial cells change so dramatically during the cell cycle that it was quite possible, without utilizing ^{3}H-thymidine autoradiography, to

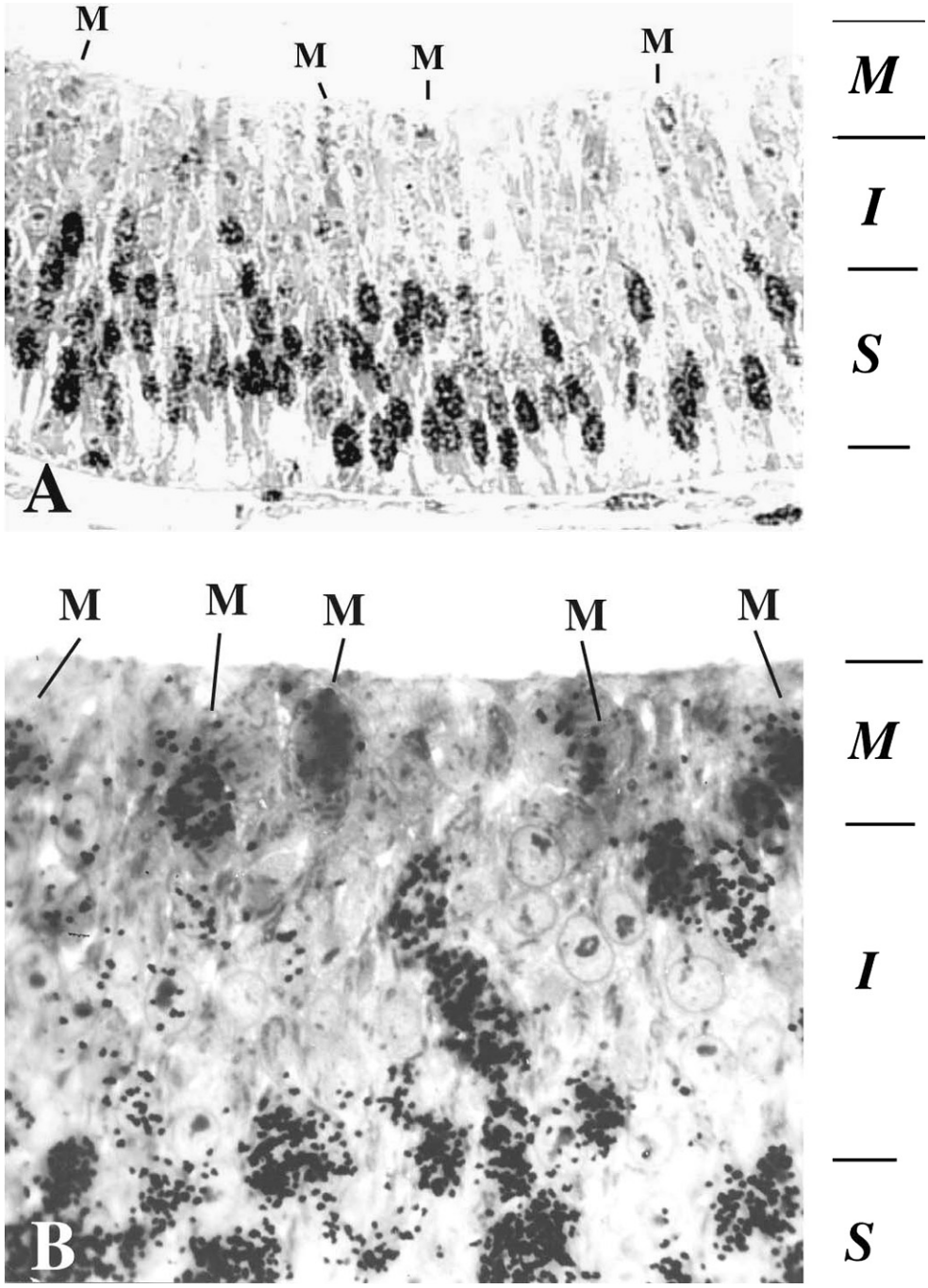
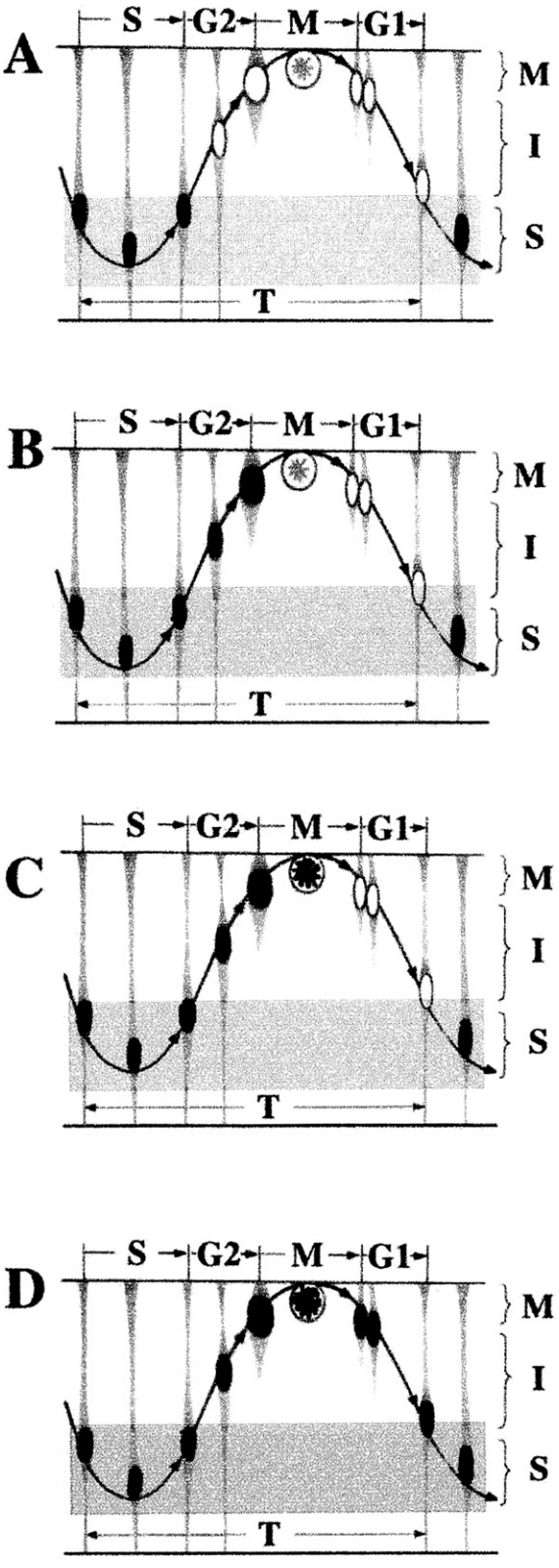


Fig. 4. ^{3}H-thymidine autoradiographs of mesencephalon of day 4 chick embryo. (A) Autoradiograph of day 4 chick embryo mesencephalon, taken 30 minutes after injection of ^{3}H-thymidine (flash labeling). ×300. Only nuclei located in the deeper half of the wall of the neural tube (*S*, zone of DNA synthesis) are covered by silver grains indicating incorporation of ^{3}H-thymidine (i.e. DNA synthesis). None of cells in the intermediate zone (*I*) nor the mitotic cells (M, i.e., "germinal cells of His") in the mitotic zone (*M*) incorporate ^{3}H-thymidine. They cannot be independently-existing proliferating cells. (B) By 3 hr, many labeled cells have ascended to the intermediate zone (*I*) and all the mitotic cells in the *M*-zone are now covered by silver grains. ×600. It is clear that the labeled cells have moved and reach the *I*- and *M*-zones. Many cell nuclei in the *I*-zone, however, are free from the label. They are the nuclei of descending cells. Adapted from Fujita, 1962, 1963.



derive a false conclusion that the neural tube is composed of the two kinds of cells: neuroblast-producing germinal cells (ventricular cells) and glial precursors called spongioblasts (subventricular cells).

Since all kinds of neurons and glial cells were produced later from the same neuroepithelial cells composing the neural tube, I concluded that they must be multipotent stem cells of the CNS. These neuroepithelial stem cells, which performed the elevator movement, constituted the sole component of the neural tube. Such homogeneous neuroepithelial stem cells had never before been described. Therefore, for simplicity sake, a specific name for them was obviously necessary. "Germinal cells" or "spongioblasts" could not be used, and the name of "neuroepithelial cell" was not adequate since it included ependymal cells and choroid plexus epithelium together with the primitive neuroepithelial cells. So I gave them the name of "*matrix cell*", a Latin adjectival noun, meaning mother or generatrix (Fujita, 1962, 1963). The adoption of this naming made description of the cytogenesis of the CNS simpler and more precise.

6. Sequential production of neuroblasts and glia from matrix cells

The organization of the CNS is extremely complicated. It was preferable to avoid entering into particular problems of regional differences, so I selected chick optic tectum and mouse cerebral hemispheres as representative objects of developmental analysis.

Starting the labeling of ^{3}H-thymidine at various times after stage I was over, I found that neuroblasts were derived from matrix cells, migrated peripherally to the mantle and marginal layers and were characterized by an absolute lack of ^{3}H-thymidine incorporation (i.e., complete repression of DNA synthesis) (Fig. 6A, arrow). Applying this principle, the production of the neuroblasts in various regions of the brain could be analyzed in detail (Fig. 7) and was found to follow a well-organized temporal and spatial pattern (Fujita, 1964, 1967a). This stage of neuroblast production is designated as stage II or stage of neuronogenesis.

With further development of the neural tube, injected ^{3}H-thymidine could no longer be incorporated in any neuroblasts or neurons thereby revealing that neuron production had ceased there (Fujita, 1964, 1965b, 1967b). To my sur-

Fig. 5. Elevator movement is visualized by ^{3}H-thymidine autoradiography. (A) Immediately after injection of ^{3}H-thymidine, only nuclei situated in the S-zone (S) are labeled as shown in Fig. 4A. (B) 2 hr later, G2 cell nuclei in I-zone (I) become labeled. (C) At 3 hr of cumulative labeling, most mitotic figures incorporate ^{3}H-thymidine (cf. Fig. 4B). (D) Up to 8 hr, in case of day 4 chick embryo, labeling index increases linearly and reaches 100%, thereby revealing homogeneity and interkinetic elevator movement of all the matrix cells. In older embryos, it takes longer to reach 100% labeling, reflecting the elongation of the cell cycle during the development. Adapted from Fujita, 1962, 1963.

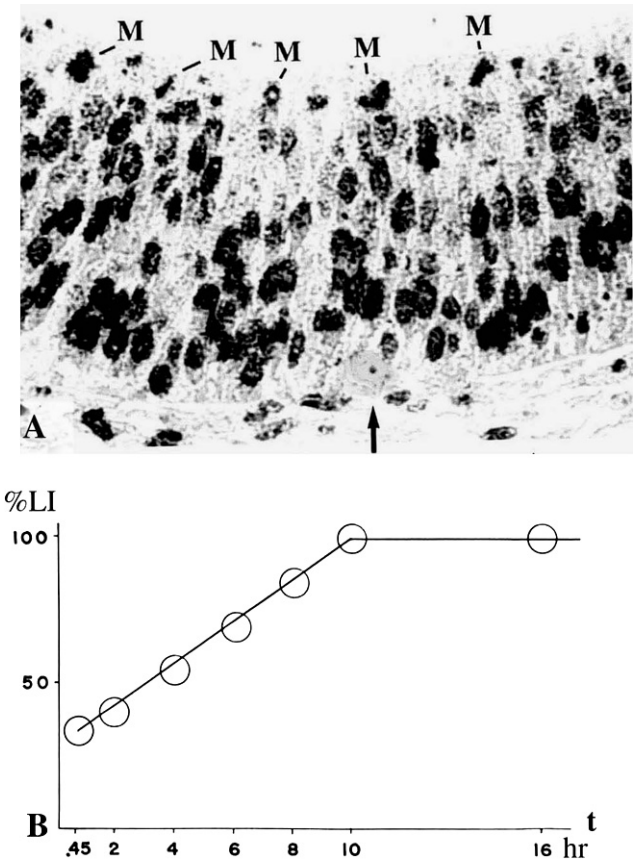


Fig. 6. Cumulative labeling with ^{3}H-thymidine at incipient stage II. (A) Chick embryo at day 4 of incubation is labeled with ^{3}H-thymidine for 8 hr (for earlier phases cf. Fig. 4). All the cell nuclei in the matrix cell layer now become labeled after a linear increase of the labeling index. It is interpreted to mean that the matrix cell layer is homogeneous at least from the kinetic point of view. ×300. Note that the first neuroblast (arrow) appearing in the mantle layer is devoid of the label notifying the beginning of stage II of cytogenesis (neuronogenesis). (B) Increase of the labeling index in matrix cell layer of 6th day chick embryo plotted against time (**t**) of ^{3}H-thymidine labeling (Fujita, 1963). The increase in labeling index in the matrix cell layer is linear as in day 4 and reaches 100% by 10 hr, thereby equally revealing cellular homogeneity of the matrix cell layer in 6th day embryo. In this material (6th day mesencephalon), the time to reach 100% of the labeling is longer than in day 4 chick embryo (8 hr), reflecting the elongation of the cell cycle of matrix cells as the development proceeds.

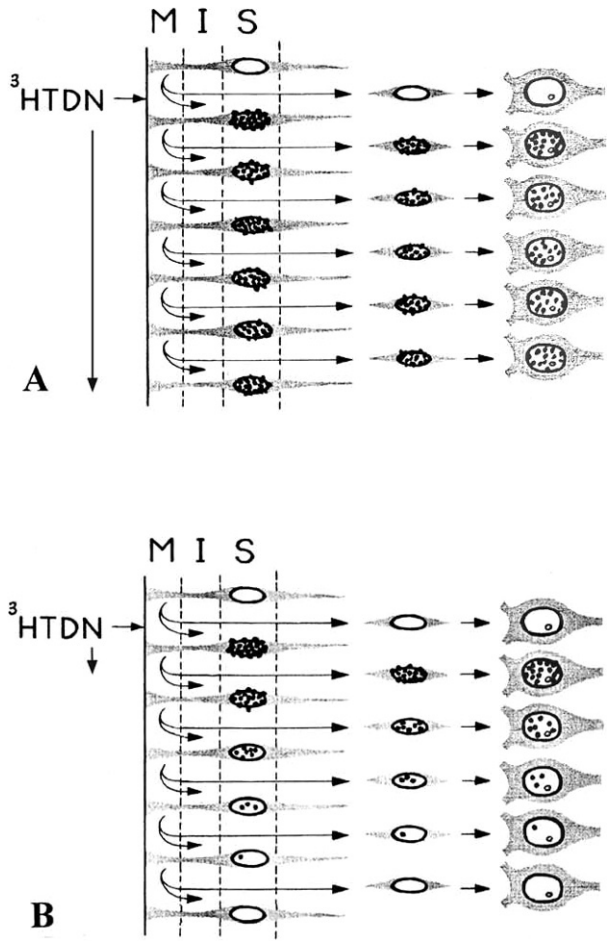


Fig. 7. Autoradiographic determination of birth dates of neurons. (A) Cumulative labeling (realized by injection in ovo, or by repeated injections). As ^{3}H-thymidine is available for a long period of time after commencement of the injection (^{3}HTDN), all the neurons produced thereafter are heavily labeled while earlier neurons remain free from the label. (B) Pulse labeling (single injection in pregnant or postnatal animals). The label is diluted by subsequent mitoses of the labeled matrix cells, the heaviest labeling is found in the neurons produced by the first division of the matrix cell, and the label in those produced by subsequent mitoses is diluted half by half until it is not recognizable. In both cases, by changing the timing of ^{3}H-thymidine injection, the birth dates of neurons from matrix cells can be analyzed in detail. ^{3}H-thymidine once distributed in the neuroblasts or neurons is expected to remain undiluted until their death so that the label in DNA serves as an excellent marker of their birth dates. This method of neuronal birth date determination is based on the fact that neurons once differentiated from matrix cells do not synthesize DNA. Adapted from Fujita, 1964.

prise, immediately following this time period, scattered (i.e., freely migrating) cells continuing DNA synthesis appeared in the mantle and marginal layers. They could be identified without doubt as progenitors of neuroglia (called *glioblasts*), since all the neurons now had absolutely ceased DNA synthesis (Fujita, 1965b). I called the three sequential stages of proliferation and differentiation of the matrix cells stage I, II and III of cytogenesis of the CNS (Fig. 8) (Fujita, 1967a, 1967b). At stage III of cytogenesis (stage of glial differentiation), ^{3}H-thymidine autoradiography revealed that the matrix cells, at the end of the stage II, switch off to neuroglial differentiation, and metamorphose into ependymoglioblasts, committed glial progenitors but morphologically indistinguishable from the matrix cells. The ependymoglioblasts are then rapidly differentiated into the ependymal cells and the glioblasts (glial stem cells). The glioblasts then differentiate astrocytes, oligodendrocytes and finally the microglia, sequentially. We regarded the microglia as the quiescent form of the glioblasts (Fujita *et*

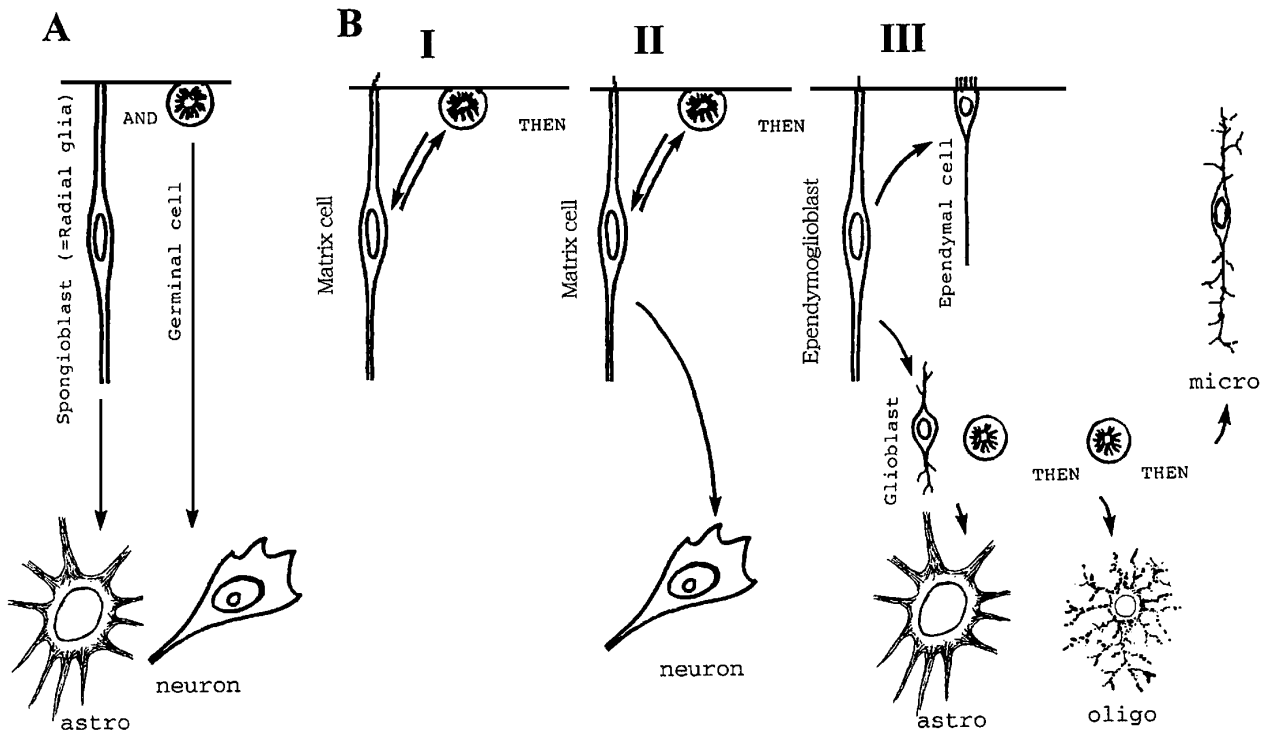


Fig. 8. Dualistic concept and the matrix cell theory on cytogenesis of the CNS. (A) Dualistic concept. Precursors of neuroglia (spongioblasts or radial glia) are differentiated early in development. Neurons are produced solely from germinal cells (syn. ventricular cells) located immediately beneath the ventricular surface. (B) Matrix cell theory, based on findings by ^{3}H-thymidine autoradiography. The course of cytogenesis in the CNS is divided into 3 consecutive stages. Stage I, pure matrix cell proliferation expanding size of stem cell population; in stage II, matrix cells begin to produce neuroblasts (stage of neurono-genesis); and in stage III, matrix cells change into ependymoglioblasts which are soon differentiated into ependymal cells and glioblasts. The gliobasts, the neuroglial stem cells, give birth to astrocytes, oligodendrocytes and microglia, in sequence.

al., 1976, 1978, Fujita, 1971, 1980, Kitamura *et al.*, 1984, Miyake *et al.*, 1984) and found that the microglia, or at least a subpopulation of the microglia, serve as glial stem cells in the adult brain when it is injured (Fujita *et al.*, 1981). In contrast, the brain macrophages are derived from circulating blood monocytes (Fujita *et al.*, 1976, 1978, 1981).

This "matrix cell theory" of cytogenesis of the CNS was adopted, replacing the thitherto widely accepted histo-genetic scheme of Bailey and Cushing (Bailey and Cushing, 1926), in Bloom-Fawcett's Textbook of Histology (Bloom and Fawcett, 1968, 9th edition, pp 344–347), with my illus-tration of the elevator movement.

7. Does a neuron replicate DNA?

The point I claimed from the autoradiographic finding that neurons once differentiated from matrix cells do not synthesize DNA met with strong criticism, at the La Jolla Symposium in 1968. Many participants of the Symposium attacked this point, utilizing the unanimous conclusions of more than 20 papers on Feulgen cytophotometry so far pub-lished from all over the world that neurons do synthesize DNA (some representative papers are Brodskij and Kusc, 1962, Müller, 1962, Mendelsohn, 1966, Sandritter *et al.*, 1967, Lapham, 1968) and contended that ^{3}H-thymidine autoradiography was not reliable. Saying that "^{3}H-thymi-dine autoradiography cannot detect neuronal DNA synthe-sis, and the matrix cell theory is developed on this method-ology", the Symposium chairman Professor S. W. Kuffler (Harvard) solemnly made the concluding remark, "There-fore, it is clear that the matrix cell theory is not acceptable."

This point was crucial: If neurons continued to synthesize DNA and ^{3}H-thymidine autoradiography failed to reveal this fact, it was clear that autoradiographic evidence to support the "matrix cell theory" did not hold, and that the theoretical basis of the birth date analysis of neurons by ^{3}H-thymidine autoradiography would also be totally lost. I was forced to challenge the worldwide conclusion of cyto-photometry of neuronal DNA-synthesis.

After 6 years of research on the methodology, I found that all the previous investigators of absorbance cytophotometry had failed to recognize the presence of non-specific light loss due to a random scatter of light when it passed through the neuronal soma very rich in protoplasm (Fujita, 1974). Scanning the object (neuronal nuclei stained with Feulgen dye) consecutively with two wavelengths, one (550 nm)

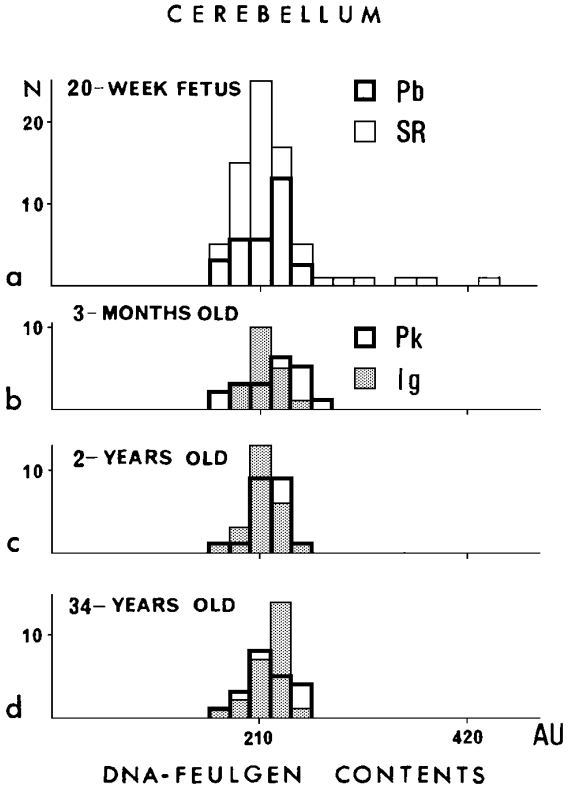


Fig. 9. DNA constancy in neurons as revealed by cytophotometry. DNA contents of voluminous cells like neurons can only be determined correctly after eliminating nonspecific light loss due to random scatter of light in the protoplasmic structure of the cell. "Two-wave-lengths scanning method" on carefully prepared cells reveal the true DNA content of neurons in the human CNS to be constantly diploid (Fujita, 1974). N, number of cell measured; Pb, neuroblast of Purkinje cell; SR, small round nuclei of external granule cell; Pk, Purkinje neuron; Ig, internal granule neuron; AU, specific absorbance in arbitrary unit.

specific for Feulgen absorbance and another (450 nm) nonspecific, that is not absorbable, it was confirmed that the voluminous protoplasmic structure rich in cell organelles of neurons scattered the light of both wavelengths in a very similar fashion, and that a significant part of the energy of the "specific" light was also lost nonspecifically. A slight adjustment of the calibration of the scanning curve at 450 nm could make the scanning curve over the extranuclear protoplasmic area perfectly overlap with that of 550 nm. After the adjustment, the scanning curve at 450 nm could be regarded as representing the nonspecific light loss in the measurement of Feulgen absorbance. Subtraction of the nonspecific light loss from the total absorbance of the "specific" wavelength (550 nm) gave constant value of 2n of neuronal DNA content irrespective of the size, kind or age of any neurons (Fig. 9). For this "two-wavelength scanning cytophotometry", an automatic cytophotometer to scan with two wavelengths was developed in collaboration with Olympus Optical Co., Tokyo.

As this finding was also confirmed by fluorescent measurement of DNA content (cytofluorometry), I reported the results in a paper entitled "DNA constancy of neurons of the human cerebellum and spinal cord" in J. Comp. Neurol. (Fujita, 1974). Thereafter, no paper ever appeared to claim DNA synthesis in neuroblasts or neurons. I was thus able to confirm that ^{3}H-thymidine autoradiography had indeed revealed the true aspect of DNA synthesis of cells in developing embryos.

8. When are neuroglial cells differentiated?

During 1969 to 1981, several investigators claimed that, in stage II of cytogenesis in the cerebral hemisphere, a second population of committed glial progenitors existed at the same time with the neuronal progenitors (ventricular cells). The former were called "subventricular cells" (Boulder Committee, 1969). Those investigators pointed out the presence of mitotic figures (Fig. 10A) in the "subventricular zone" (i.e., at some distance from the ventricular surface) (Fig. 10A, *I*) as evidence: The subventricular cells do not perform the elevator movement thereby revealing their nonmatrix cell nature. "If they do not belong to the matrix cells", those investigators concluded, "they must be glial cells", though there was no positive evidence for their neuroglial nature, neither in morphology nor by specific glial staining.

I could not agree with this point, because I had found vigorous incorporation of ^{3}H-thymidine by vascular cells forming the capillary network in the very same zone from a very early stage of neuroblast production (Fig. 10B). These mitotic figures are, in fact, nothing other than those of vascular cells. Those investigators failed to recognize the fact that stage II of cytogenesis is always accompanied by an intramural invasion of blood vessels, and that immediately thereafter the most active vascular growth takes place. Strong (1964) studied development of vascular networks in the so-called subventricular zone by india ink infusion method in stage II of rabbit cerebral hemispheres and observed rapid growth of vascular systems in the wall during neuronogenesis.

The Boulder Committee (1969) had ignored the existence and growth of vascular networks at stage II of cytogenesis in the subventricular zone of the cerebral hemisphere, which supply the blood for the rapidly enlarging brain tissue.

9. Production of glial cells follows that of neurons

Although this debate on the vascular nature of the subventricular mitoses had not been settled, a new criticism based on histochemistry of GFAP (glial fibrillary acidic protein) appeared: Several investigators (Antanitus *et al.*, 1976; Levitt *et al.*, 1981; Choi, 1986) began to report strong reactions of anti-GFAP antisera in some cells in the matrix cell

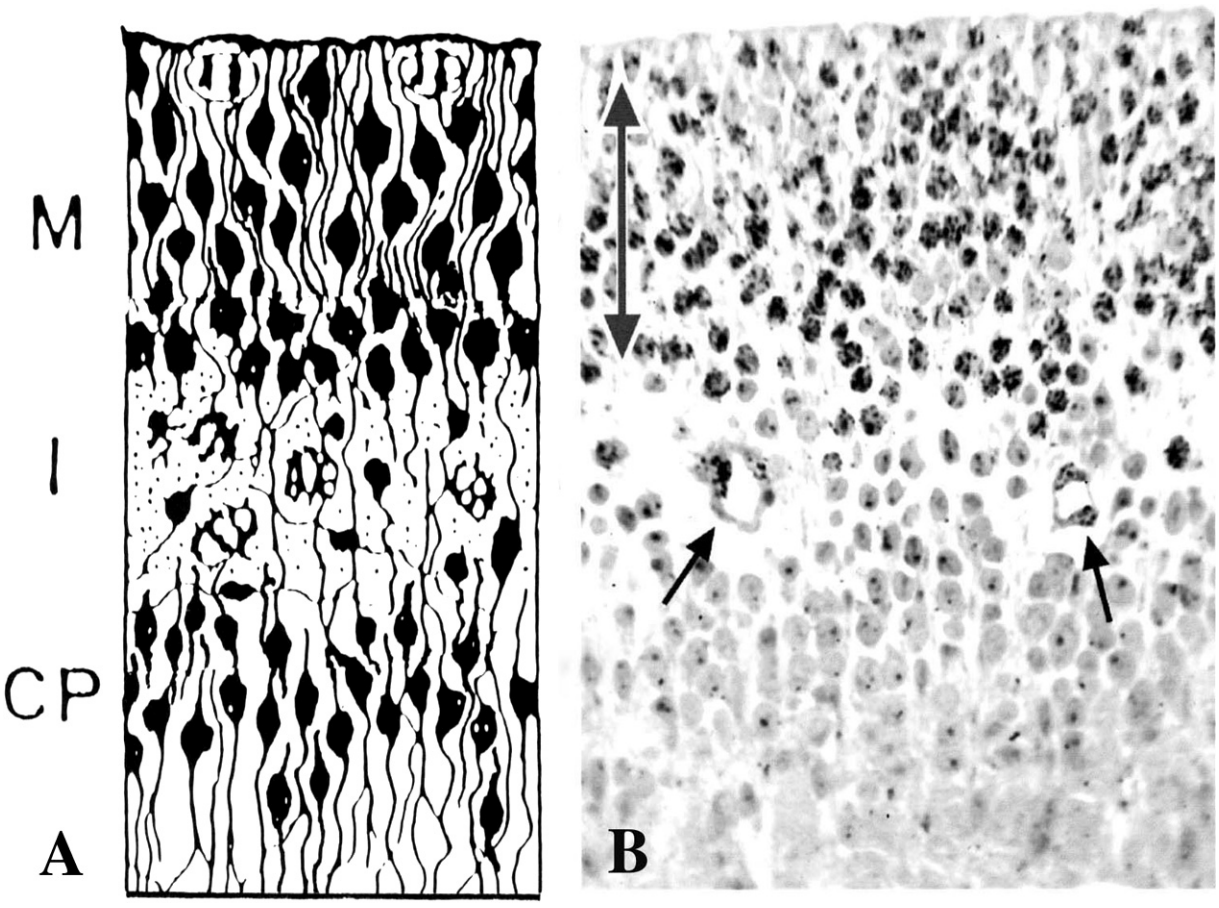


Fig. 10. Proliferation of vascular cells in the subventricular zone during stage II. (A) Schematic drawing of wall of cerebral hemisphere during stage II (adapted from Boulder Committee, 1969). Many mitotic figures appear in the subventricular zone (I) subjacent to the matrix cell layer (M). In contrast to the mitoses of the matrix cells, which divide directly beneath the ventricular surface (top of the scheme), the presence of the deep mitoses reveals, the Committee members concluded, that these cells do not perform the elevator movement, and that they are different kinds of cells from the matrix cells. The Committee inferred that they must be glial progenitors, without any other positive evidence. (B) However, cumulative labeling with ^{3}H-thymidine of the chick mesencephalic wall at incubation day 6 (stage II, i.e., under active neuronogenesis) for 10 hr reveals the presence of actively growing capillary network in the subventricular zone in which many vascular cells incorporate ^{3}H-thymidine (two arrows), while the cells (neuroblasts) accumulated around and below this zone (mantle layer, CP) are totally unlabeled. A few neuroblasts, which have inherited ^{3}H-thymidine several hours earlier from their mother matrix cells now begin to migrate down into the upper intermediate zone. ×200. The Boulder Committee (1969) paid no attention to the presence of the blood vessels in the brain.

layer in the cerebral hemisphere at an early stage of development, and claimed that neuroglial differentiation proceeds parallel with the production of neurons.

Rakic (1972) and Levitt *et al.* (1981) named them "radial glia", and this concept and nomenclature spread rapidly among neuro-embryologists. This claim, however, of the presence of a GFAP-positive second population in the matrix cell layer contradicts our previous observations: Firstly, cumulative labeling of ^{3}H-thymidine autoradiography excluded the possibility of the heterogeneity of the matrix cell layer, and secondly, electron microscopic observations of the neural tube (Fujita and Fujita, 1963, Fujita, 1967b) did not allow for such a dualistic interpretation.

If some cells in the matrix cell layer produced such great amounts of GFAP, the relationship of the GFAP-positive population to the matrix cells in terms of neuroglial differentiation would have to be re-examined. Those investigators who observed the positive GFAP reaction used antisera provided by Eng, or by Bignami and Dahl (1974). Curiously enough, Eng *et al.* (1971) as well as Bignami (1991) had never observed any positive GFAP reaction in matrix cells (neuroepithelial cells) at stages I and II with their own antisera. Critical re-examination was obviously necessary.

Eng, and Bignami and Dahl kindly provided us with the antisera they had raised, and we could carry out comparative study with our own anti-GFAP antibodies. We investigated chick, mouse, rat, bovine and human fetal brains and spinal cords by applying immunohistochemical staining, chemical analysis with SDS-PAGE, and immunoblotting to detect occurrence of GFAP (Fig.11) (Fujita *et al.*, 1981,

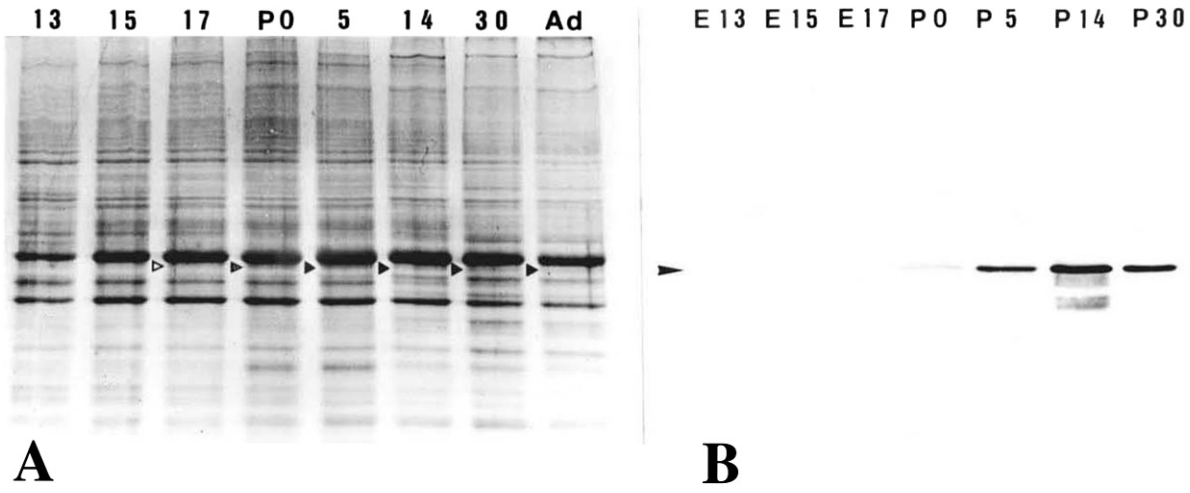


Fig. 11. Absence of GFAP during stage II of cytogenesis. (A) SDS polyacrylamide gel electrophoresis (PAGE) of mouse cerebral cortex at various developmental stages (Embryonic days 13 to 17, postnatal days 0 to 30 and Adult), and (B) its immunoblotting for Glial Fibrillary Acidic Protein (GFAP). Adapted from Fujita *et al.*, 1985, 1986. During stage II of cytogenesis in the mouse (E 13–17), no GFAP is detectable. Similar findings have been repeatedly obtained on chick optic tectum, rat, calf and human cerebral hemispheres. Recent investigation has been extended to include mRNA detection by Northern blotting, *in situ* hybridization, RT-PCR, and the results unanimously confirm that GFAP is neither expressed nor transcribed during stages I and II of cytogenesis (data not shown).

1985), together with detection of GFAP-encoded mRNA by *in situ* hybridization and Northern blotting (Fujita *et al.*, 1986). We came to the conclusion that GFAP and its mRNA are not present in stage I and II matrix cells, at least not at a level detectable using the current techniques. Using probes for GFAP-mRNA, Lewis and Cowan (1985) reported similar observations in the mouse and Capetanaki *et al.* (1984) in chick brains. These authors did not detect any positive signal throughout stage I and II of cytogenesis, but a strongly positive signal appeared when stage III began in those regions. Recently Nakamura, Y. (submitted for publication, 2003) obtained the same results as ours by Western blotting and RT-PCR on human fetal brains. These results confirmed that the occurrence of GFAP-encoded mRNA parallels the appearance of GFAP molecules in the developing CNS, and that the GFAP-encoded mRNA never becomes detectable during stages I and II of cytogenesis (Fujita, 1986). Thus I concluded that, from the morphological, histochemical, and kinetic points of view, during stages I and II the matrix cell layer was composed of a basically homogeneous population of stem cells, though some migrating neuroblasts could temporarily be intermingled among them.

This conclusion of the homogeneity of the matrix cell layer was important for me to analyze cell proliferation kinetics in various parts of the brain, because to calculate cell cycle parameters by ^{3}H-thymidine autoradiography the assumption of the homogeneity of object population was essential (Fujita, 1962), since most analytical methods of cell proliferation kinetics could not be applied to a heterogeneous object population.

10. Regional differences of matrix cell differentiation in the CNS

There are marked regional differences in the progressions of the stage from I to II, and from II to III in various parts of the CNS. In some regions, neuronogenesis (stage II) is prolonged and the transition between stage II to III takes place after birth: (1) In CA3 and fascia dentata of hippocampus (Altman and Das, 1965a, 1965b, Cameron *et al.*, 1993, 2001), the association-granule neurons are produced well into adult life. (2) Olfactory association-neurons continue to be produced postnatally from stem cells in the ependymal (=ventricular) layer of the anterior part of the lateral ventricle in the rodent (Altman, 1969, Johannson *et al.*, 1999), which possess characteristics of epithelial stem cells having single cilium and performing the elevator movement (Tramontin *et al.*, 2003). The neuroblasts produced from them migrate rostrally along the rostral migration stream (RMS) to the olfactory bulb and integrate into the synaptic circuitry in the brain (Carlen *et al.*, 2002). (3) In the cerebellum, the matrix cells of the rhombic lip are detached from the ventricular surface, migrate subpially to the external cerebellar surface, spread there as external matrix cells and give birth to internal granule neurons for sometime after birth (Fujita *et al.*, 1966, Fujita, 1967a). These postnatal neurogeneses in the hippocampus and cerebellum, however, are reduced as the animals grow older (Zitnik and Martin, 2002). On the other hand, as a continuation of postnatal neuron production, some of the matrix cells persist longer than the physiological neuronogenesis, as long-persistent or remnant "neural stem cells", among nestin-positive ependymal cells

(Johannson *et al.*, 1999). It is reasonable to assume that these remnant matrix cells in the ependymal layer, as a continuation of the pre- and postnatal neurogenesis, retain their potential to contribute to neuronal and glial production when they receive appropriate proliferation signals (Johannson *et al.*, 1999).

In songbirds, seasonal neuron reproduction is known to occur from certain ventricular cells in a special sector of the caudal forebrain during their entire life span (Nottebohm, 1985). They are identified as multipotent epithelial stem cells (matrix cells) , which remain in the adult ependymal layer performing the elevator movement (Alvarez-Buylla *et al.*, 1998, Louissaint *et al.*, 2002).

11. Molecular mechanism of the elevator movement

To understand the mechanism of the rounding off of the mitotic epithelial cells, it is essential to understand the molecular mechanism of epithelial cells that assures their morphological property of single-layered cylindrical epithelium. Cell-to-cell and cell-to-connective tissue adhesions are particularly important in epithelial morphogenesis. Common to all the single-layered cylindrical epithelia in various organs, cell surface adhesion apparatuses like tight junctions, adherent junctions, desmosomes, and adhesions to extracellular matrix (ECM) are present at adherent sites to the neighboring cells and to ECM, and play important roles in keeping the elongated epithelial morphology of the

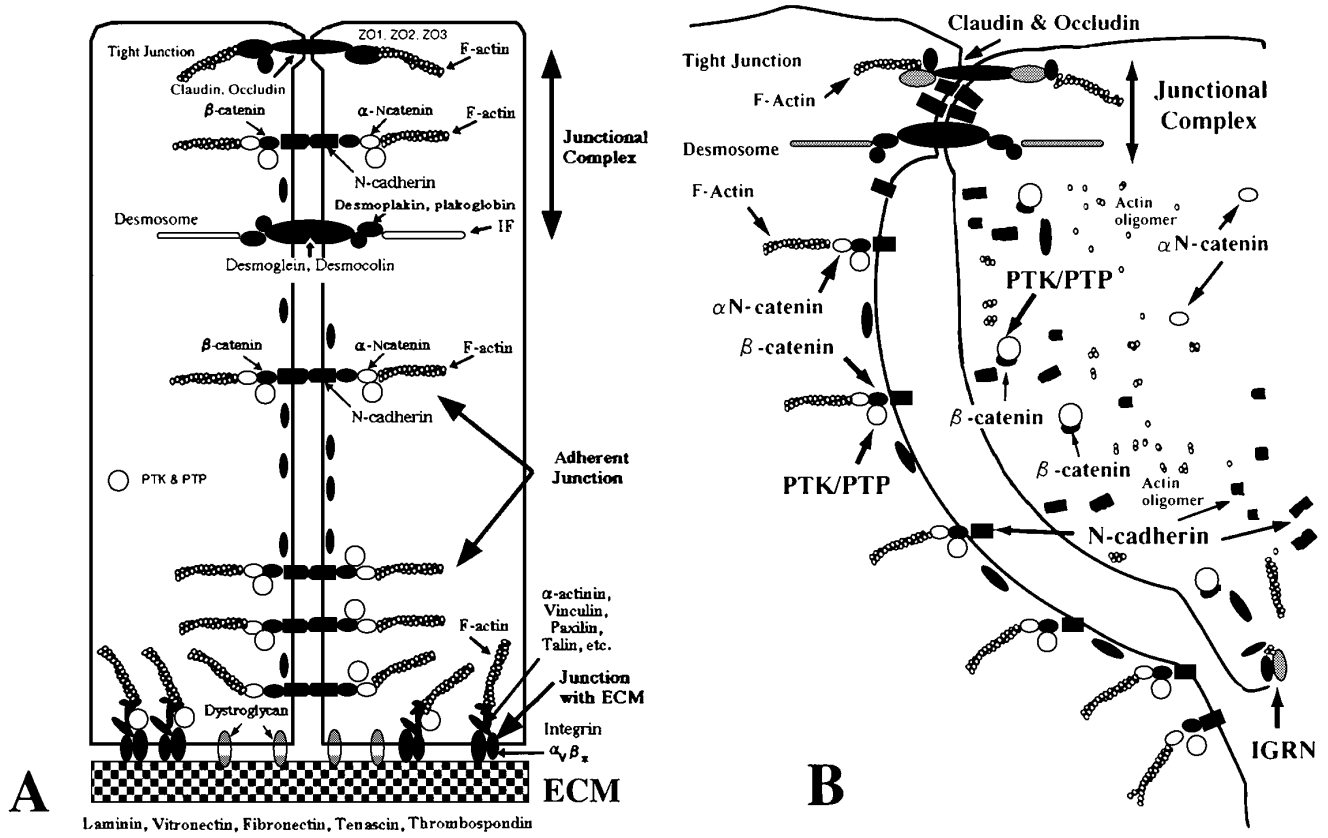


Fig. 12. Molecular mechanism of the elevator movement. (A) The single-layered epithelial morphology of the matrix cells in the vertebrate CNS is maintained by three types of adhesion apparatuses: junctional complexes, adherent junctions and adhesions with ECM (extracellular matrix). Integrin, which links basal cell membranes with ECM, is a heterodimer composed of two subunits, α and β, which comprise several subtypes. In matrix cells, α_V integrin has been shown to be essential, but the exact nature of its partner (shown here as $\beta_\star$) is not known. Among the various ECM molecules (listed below the basement membrane represented by checkerboard), dystroglycan is an odd member. It is produced by matrix cells, secreted from the matrix cell and is cleaved into 2 moieties, α and β. One moiety of the molecule, α-dystroglycan forms a link between the rest of the molecule, β-dystroglycan, and initiates basement membrane assembly. IF, intermediate filament; PTK, protein tyrosine kinases; PTP, protein tyrosine phosphatases. (B) Mitotic changes of intracellular distribution of the adhesion molecules. During mitosis, aggregates of the N-cadherin-β-catenin-α-catenin-F-actin complex are dispersed and the components dissociate, thereby disrupting cell-cell adhesion. Simultaneously, or sometimes lagging behind time, the release of adhesion of integrin (IGRN) from ECM takes place. Adapted from Fujita and Yasuda, 2003.

cell (Fig. 12A).

Molecular analysis reveals that adherent junctions are cell-to-cell adhesion sites where classic cadherins gather as cell adhesion molecules and where the actin-based cytoskeleton and several cytoplasmic factors are assembled (Takeichi, 1977). It is now widely accepted that intracytoplasmic regions of cadherins are directly bound to β-catenin, which in turn binds to α-catenin; α-catenin then binds to actin (Fig. 12A) (Imamura *et al.*, 1999). These chain formations of extracellular cadherins with intracellular catenins are particularly important molecular events to establish and maintain cell adhesion.

When β-catenin is phosphorlylated and loses the binding affinity to cadherin and α-catenin, however, the homophilic intercellular binding affinity of the cadherin is totally lost (Hirano *et al.*, 1992). This is a key reaction to regulate cadherin interactions between adjacent cells in a completely reversible fashion.

The junctional complex is a pivotal cell-to-cell adhesion apparatus composed of tight junction, adherent junction and desmosome. The junctional complex is also called a terminal bar when observed under light microscope. This complex is the most stable adherent apparatus that resists strong mechanical shearing forces. Another important characteristic of the junctional complex is that it persists during mitosis while other adherent apparatuses appear to be lost transiently, if not completely, during the entire processes of mitosis (Fujita and Fujita, 1963).

Epithelial cells are firmly attached to the basement membrane, not only in adult tissues but also in embryonic development. In the formation of the basement membrane, α- and β-dystroglycans are secreted from the epithelial cells as core molecules of the basement membrane and the α-dystroglycan molecule anchors the cell onto the basement membrane (Fig. 12A) (Helmer, 1999). Laminin in the basement membrane binds to epithelial basal surface receptors such as α and β integrins (Fig. 12A). Like cadherins that bind lateral cell membranes, the binding affinity of integrins to the basement membrane is regulated by conformational changes of the integrin caused by multi-protein complexes bound to the intracytoplasmic region of the integrin (Xiong *et al.*, 2001).

Our immunohistochemical observations on changes in distribution of adhesion molecules during mitotic cycle unveiled the molecular mechanism of the elevator movement (Fujita and Yasuda, 2003): Adhesion apparatuses of N-cadherin-catenin-actin complexes dissociate during mitosis releasing the intercellular adhesion from the neighboring cells. Simultaneously, the release of adhesion of the integrin systems takes place and the mitotic matrix cells are rounded off and inevitably attracted to the junctional complexes on the ventricular surface (Fig. 12B). After division, the daughter cells recover the N-cadherin-β-catenin-α-catenin-actin complexes and their function of intercellular adhesion and resume their downward elevator movement.

12. Molecular mechanism of radial migration of neuroblasts

When a neuroblasts is differentiated from the matrix cell, two characteristic changes occur, other than the complete repression of DNA replication: (1) Loss of adhesion on the ventricular surface with junctional complexes followed by atrophy of the apical process (Fig. 13A, small arrow), and (2) the emergence of axon from the basal process which loses adhesion to ECM (Fig. 13A, Gc). The simplest explanation of these two changes is to assume the switching off of constituent proteins in the junctional complexes and integrin adhesion systems in the neuroblast (Fig. 13B). The detailed molecular mechanism of this down-regulation of the two systems is to be elucidated in the future. When these two adhesion systems are switched off, however, the adherent junctions of the neuroblast to the adjacent matrix cell processes (or bundles) are conserved, and the adherent junctions move distally as the neuroblast migrates out of the matrix layer. Therefore, the neuroblasts begin their migration by exactly the same mechanism as the downward elevator movement (of matrix cells). As they have lost their apical attachment to the ventricular surface that has served as the anchorage of the cells to keep them within the limit of the matrix cell layer, they begin to migrate freely along the elongated processes (or bundles) of neighboring matrix cells until they move out into the subpial position, forming the mantle layer or cortical plate (Fig. 13A, B).

The distal ends of the neurites (axons) including growth cones are rich in factors of the multi-protein cadherin systems. The axons can proceed in the subpial layer, where they extend their growth cones, which adhere to the distal processes of matrix cells and the preceding axons. These axons usually run in a direction perpendicular to the axis of the matrix cell processes.

13. Bundle formation of the matrix cells has important functions in corticogenesis

Throughout stage II of cytogenesis in the cerebral hemispheres of human fetuses, mouse embryos and the mesencephalic wall of the chick embryos, the distal cytoplasmic processes of matrix cells are found to form bundles that run radially throughout the entire thickness of the brain wall (Fig. 14). Bundle formation occurs due to the fact that the lateral cell membranes of matrix cells in the corticogenetic region adhere to each other by the N-cadherin system. Since the bundles are made up of the processes of many matrix cells ranging from 5 to 50 (10–20 on average), they can be maintained as stable structures despite the periodic (but asynchronous) withdrawal of individual processes due to the elevator movement.

The functions of the bundles are threefold (Fig. 15A) (Fujita, 1986). First, they guide young neurons in their migration into the cortical plate that lies distant from the

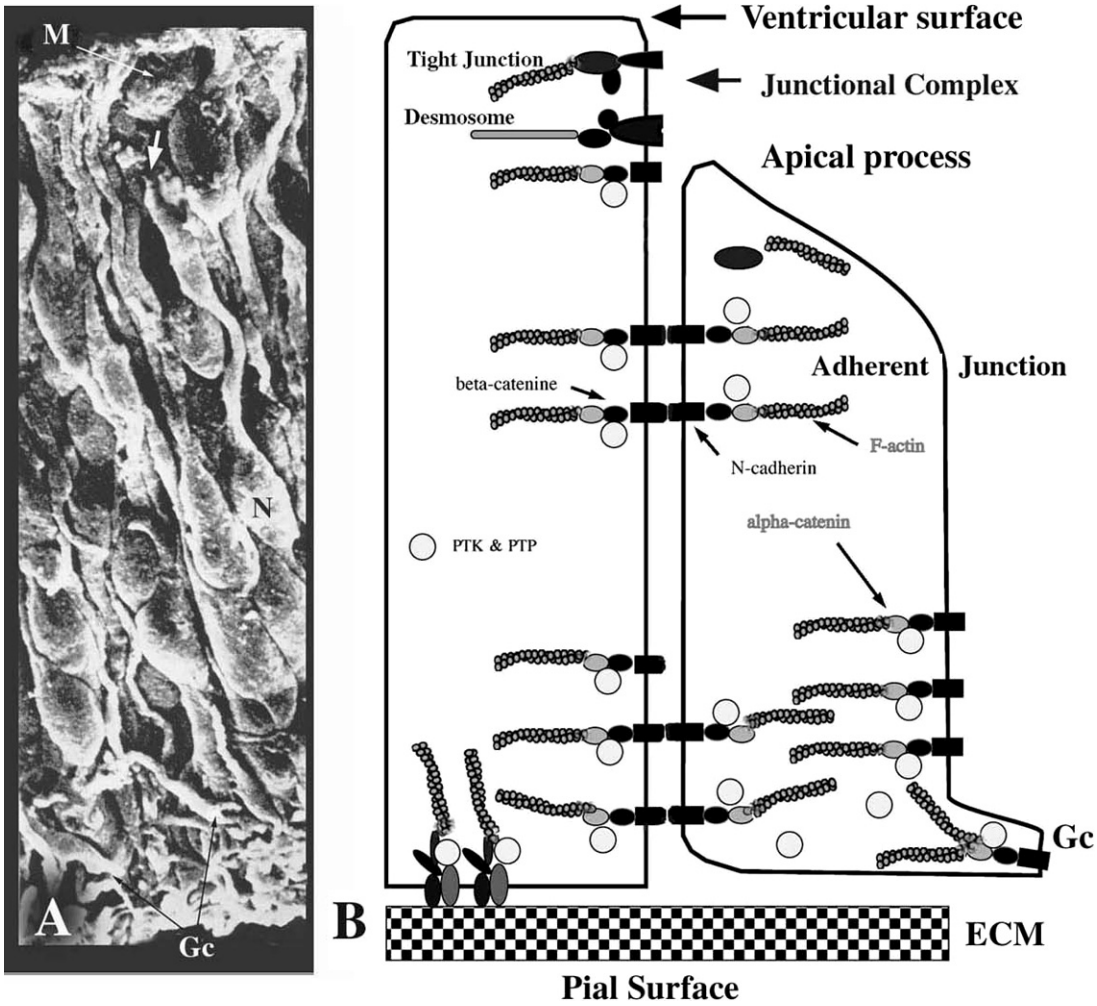


Fig. 13. Molecular mechanism of migration of neuroblasts. (A) SEM fractograph of neural tube at incipient stage II of cytogenesis. The mesencephalic wall of a 3-day chick embryo shows the beginning of neuroblast differentiation. ×1,020. Note that the apical process (short arrow) of a newly formed neuroblast (N) is released from the luminal surface as a result of down-regulation of the junctional complex proteins. Growth cones (Gc) of newly emerged axons begin to run parallel with the pial surface in the deepest part of the neural tube. M indicates rounded mitotic matrix cell. (B) Changes in cell-adhesion organization at neuroblast differentiation. Upon differentiation into neuroblasts, matrix cells lose both junctional complexes and adhesions with the ECM. Only adherent junctions between the newly formed neuroblasts and adjacent matrix cells are conserved, and most of the adherent apparatuses move to the distal end of the neuroblast, resulting in an increase in adhesive potency in the distal portions of the neuroblast (growth cones, Gc) which pull the neuroblast cell body during subsequent migration.

matrix cell layer, to form a unit structure of the cortical column. For this function, the dynamic adhesion of neurons to matrix cells undoubtedly plays an important role. The second function of the bundle is to guide the processes of matrix cells themselves during their elevator movement. Without these guide rails, the smooth performance of the elevator movement in so thick a wall like cerebral hemisphere or mesencephalic wall of chick would be extremely difficult. The third function of the bundles of progenitor cell processes becomes manifest at the end of stage II of cytogenesis. When the bulk of neuron production is completed in the corticogenetic area, the matrix cells are differentiating into ependymogliobasts (common ependymal and glial precursors), which are rapidly differentiated into gliobasts, as shown in Figure 16. The rest of the ependymoglioblasts are changing into single-layered ciliated ependymal cells.

In some particular regions, however, such as the rostral part of lateral ventricles of mammals where the olfactory neurons are produced (Altman, 1969) or in a specific sector of the neostriatum of songbirds (Nottebohm, 1985) where neurons of higher vocal centers are reproduced seasonally, the matrix cells are suppressed in their progression from stage II to stage III for extended periods of time, so that the residual matrix cells persist postnatally in the ependymal

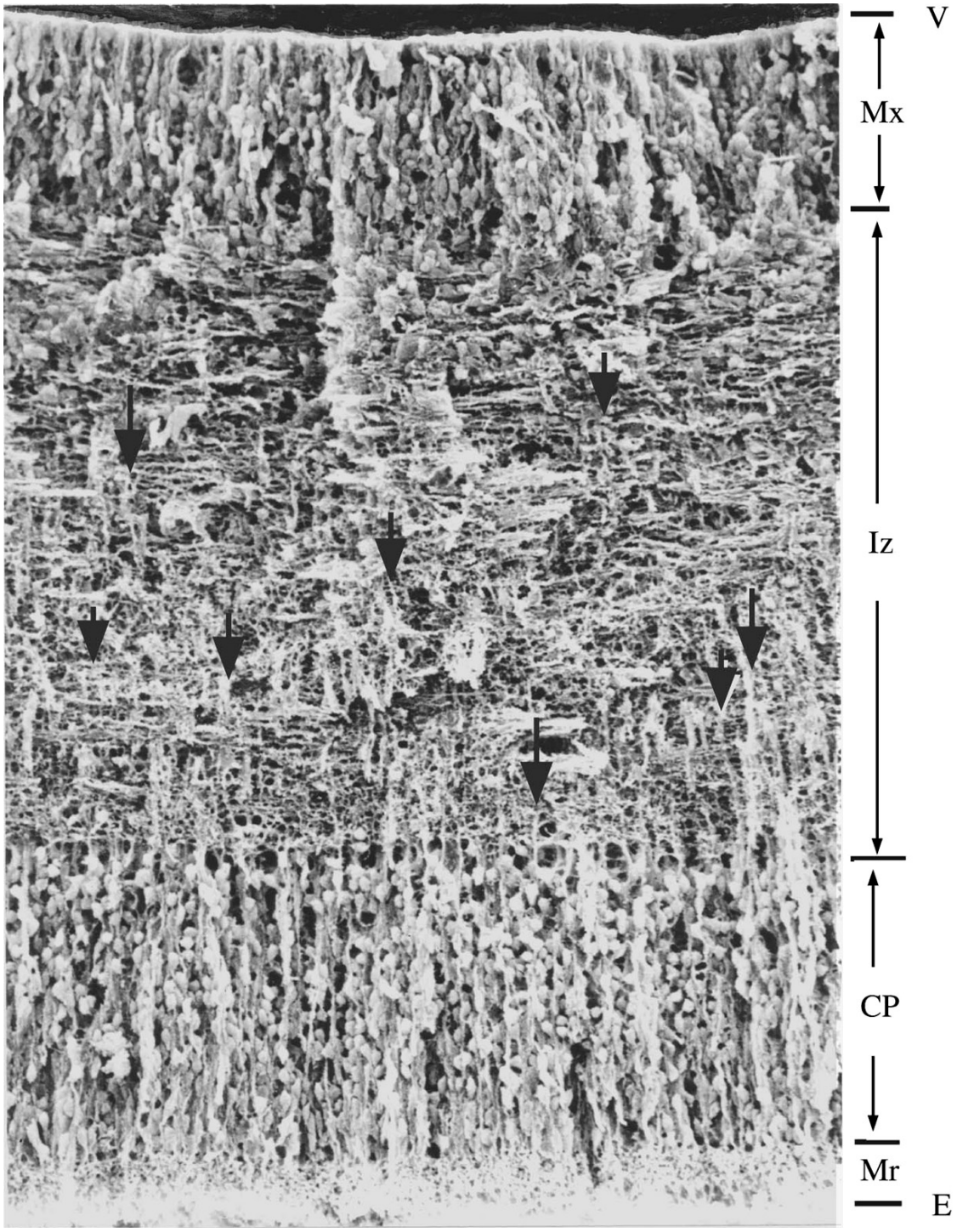


Fig. 14. SEM fractograph of the cerebral hemisphere of a 12-week human fetus. The matrix cell layer (Mx) is at the top, and the cortical plate (CP) and the external surface of the brain (E), at the bottom. Processes of the matrix cells form bundles, which traverse the entire thickness of the cerebral wall. Neuroblasts are attached to the bundles and migrate into columns of the cortical plate. Arrows indicate bundles of matrix cells. ×250. V, ventricular surface; Iz, intermediate zone; CP, cortical plate; Mr, molecular layer.

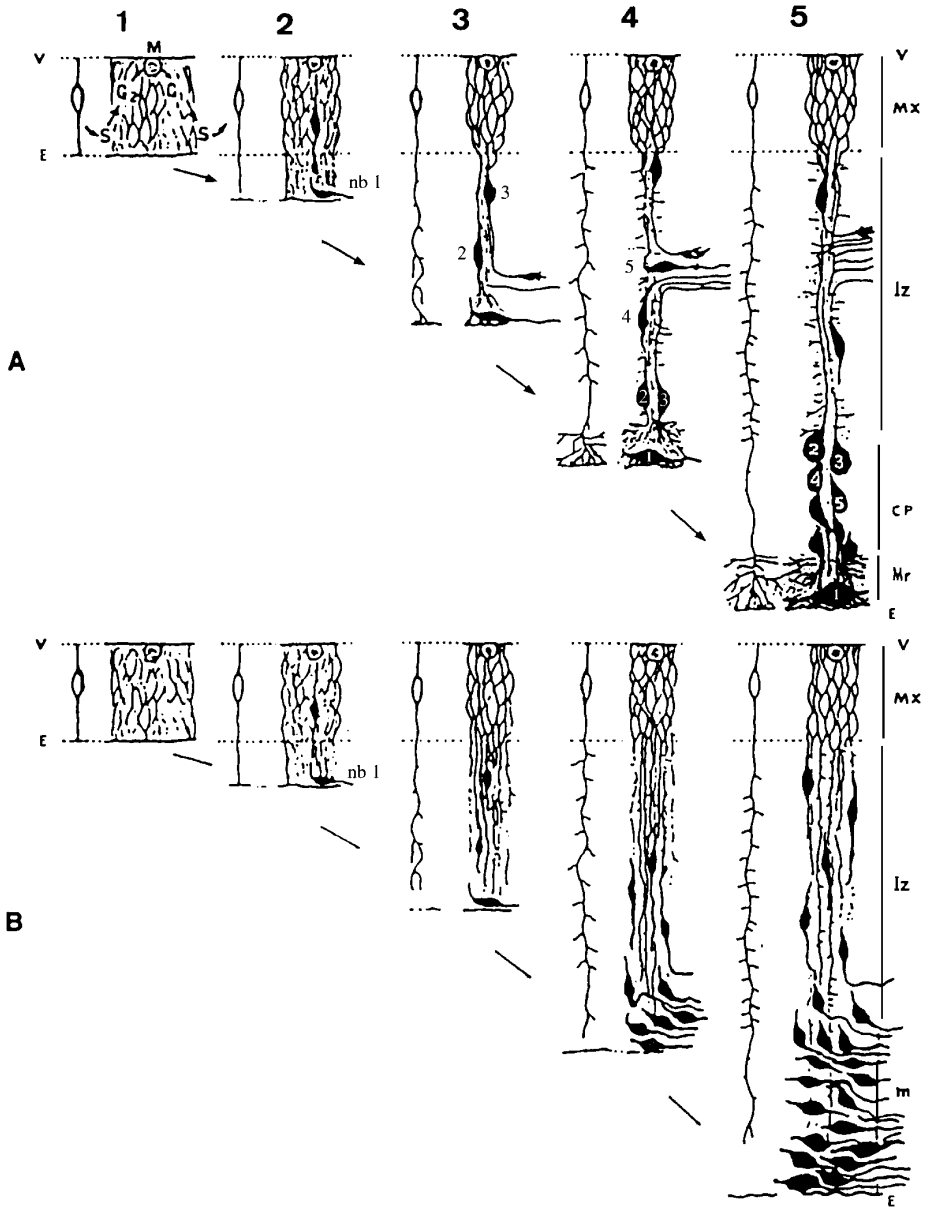


Fig. 15. Generation of cortical neurons in an inside-first, outside-last gradient and histogenesis in non-corticogenetic region. (A) Keeping pace with thickening of the wall of the brain (from left to right), matrix cells elongate, their processes tend to adhere to each other and form bundles (**1** to **5**). **1.** Stage I of cytogenesis. **2.** The first neurons produced in the matrix cell layer (Mx) before the bundles are formed (i.e., Cajal-Retzius neurons, nb l) migrate to the subpial position and become embedded in the molecular layer (Mr). **3.** Subsequent neurons (nb 2, nb 3) produced from the same group of matrix cells that now form a bundle migrate along the bundle to the cortical plate (CP) and reach the deepest position adjacent to the molecular layer. **4**–**5.** Subsequently generated neurons (nb 4, nb 5) migrate past the older cells, progressively forming the more superficial cortical layers. The older neurons are schematically illustrated as larger cells. Evidence from cell lineage experiments indicates that clonally related neurons can span several cortical layers, suggesting that precursor cells divide many times to produce a series of neuroblasts during stage II. (B) Histogenesis in the non-corticogenetic region. Matrix cells in this region do not form bundles. As the wall thickens, many matrix cells do not reach the pial surface (E) and the molecular layer is not formed. Neuroblasts migrating along the individual processes of matrix cells are dislodged as the latter engage in the elevator movement. The neuroblasts become piled up in the mantle layer (m) in an order reflecting their birth times, resulting in the creation of an "outside-in pattern". V, ventricular surface; Mx, matrix cell layer; Iz, intermediate zone; CP, cortical plate; Mr, molecular layer, E, external or pial surface; nb, neuroblast; nb1, Cajal-Retzius cell; small numbers (nb)2–5 indicate cortical neuroblasts 2 to 5 produced following Cajal-Retzius cell in sequence. Adapted from Fujita, 1986.

layer intermingled with the ependymal cells. These postnatal neural stem cells, however, decrease in number as the animals grow older (Seki and Arai, 1995, Tropepe *et al.*, 1997).

The differentiation of ependymoglioblasts into ependymal cells and glioblasts is characterized by rapid reduction or loss of the long bipolar processes, which have spun between the pial and the ventricular surfaces, running perpendicularly through the cortical plate. When the processes of the matrix cells are withdrawn, they leave empty spaces or channels running perpendicularly through the cortical plate, into which thalamic fibers or other input axons, having come and waited at the entrance of the cortical plate, penetrate and form synapses with cortical neurons in the column replacing the matrix cell processes (Fig. 16). This is why everywhere in the central nervous system rapid increase of synapse formation coincides with the beginning of stage III of cytogenesis.

This is an important mechanism to make cortical columns act as functional units in the mammalian cerebral cortex. Simultaneously, cortical neuroblasts are now allowed to grow as they receive the full complement of synapses. As a result, the size of the brain parenchyme of that specific locus expands to be manifest as the gyrus. The gyrus formation in the cerebral cortex always starts sometime after the commencement of stage III of cytogenesis, in parallel with progression of the glioblast differentiation.

14. Neuron types are determined at early developmental stages

In the CNS, modification of neuronal fate can occur only at early stages of matrix cell differentiation, in response to environmental influences and following genetic switches such as the activation of position-specifying genes. However, when stage II of neurogenesis starts, neuronal fate is

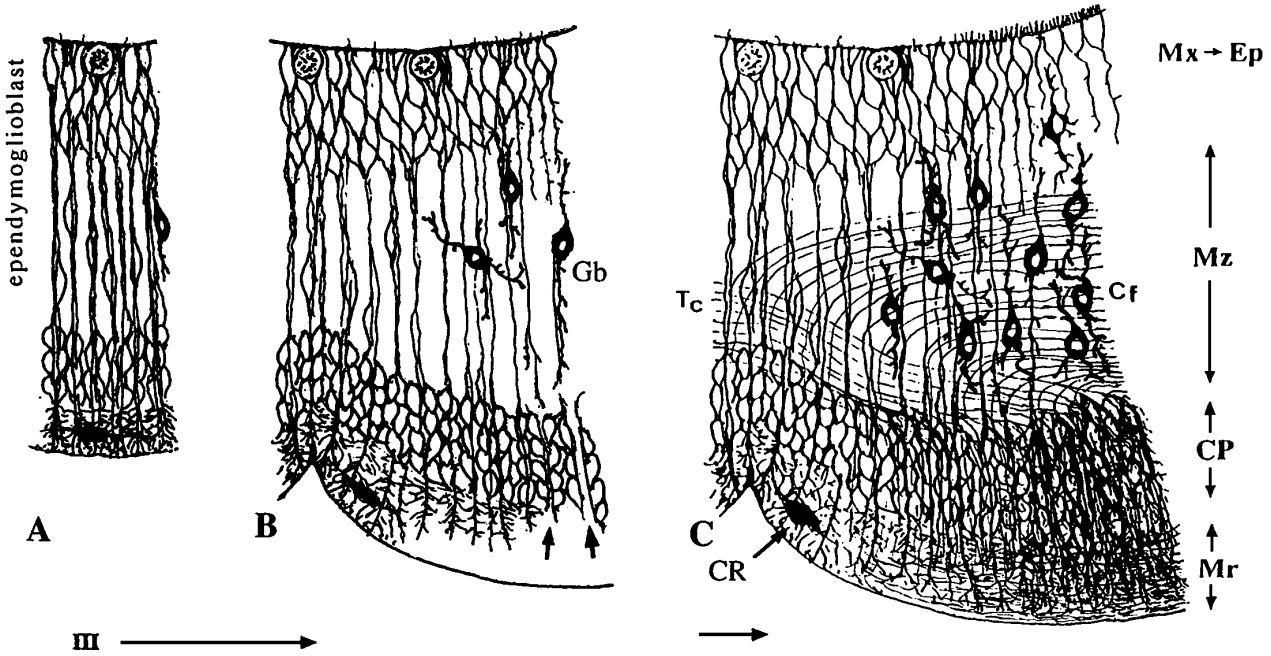


Fig. 16. Formation of cortical columns and gyri in the cerebral hemisphere. (A) Upon commencement of stage III in the cerebral hemisphere, matrix cells lose their potential for neuroblast production and transform into ependymoglioblasts, common progenitors of ependyma and neuroglia. At this stage, the walls of the cerebral hemispheres are already quite thick but still growing. Although matrix cells are now destined to become glial progenitors, they retain most of the phenotypical properties of matrix cells for some time: They continue to form bundles, to guide late neuroblasts, and perform elevator movements. (B) The gradual differentiation and migration of glioblasts (Gb) from the bundles of ependymoglioblasts result in a continual thinning of the bundles, and finally the bundles disappear when the last glioblast migrates out, leaving empty spaces (2 arrows pointing upwards) in the center of each neuroblast column in the cortical plate. (C) The rest of the ependymoglioblasts are changing into single-layered ciliated ependymal cells. In particular regions of lateral ventricles of mammals producing olfactory neurons or in a certain specific section of the hyperstriatum of birds, which gives birth to neurons of song centers, the matrix cells are delayed in the progression from stage II to stage III for some extended times, so that the residual matrix cells persist among the ependymal cells for a considerable period of time after birth, though reducing in number with time. When the radial processes of the ependymoglioblasts are lost, the axons that have come from the thalamic centers or from other cortical areas and waited in the subcortical and marginal layers now dash into these channels in the centers of cortical columns and begin to form synapses with the neuroblasts in the cortical columns. This is an important mechanism to make cortical columns as functional units in the mammalian cerebral cortex. Cortical neuroblasts are now allowed to grow as they receive the full complement of synapses, and as a result, the size of the brain parenchyme of that specific locus expands to be manifest as the gyrus. Tc, thalamo-cortical fibers; Cf, cortico-fugal fibers; CR, Cajal-Retzius neuron; Mx→Ep indicates matrix cell layer changing into ependymal layer.

irreversibly fixed. This kind of irreversible differentiation is confirmed in mutant mice (Caviness, 1982), in the transplantation of an incised piece of neural tube into heterotopic sites (Nakamura, 1990) and explantation experiments *in vitro*. Environments have failed to change the original type of differentiation of neurons in the CNS once neuronogenesis has started in the transplant. It is likely that basic type-differentiation of neurons is determined irreversibly when these cells are produced from multipotent stem cells (matrix cells) at stage II, although the same type of neuron retains a remarkable plasticity to adapt to various functional requirements.

15. Major differentiation as a basic mechanism of cytogenesis of the CNS

It is generally believed that vertebrate development, viewed at the cellular level, proceeds by sequential steps in which potencies of progenitor cells become progressively and irreversibly restricted. This type of differentiation has been dubbed major differentiation (Fujita, 1965a), as opposed to the reversible expression or repression of genes, the minor differentiation. Perhaps the simplest way to explain the mechanism by which cell potencies are restricted is to assume (Fig. 17) a progressive accumulation of irreversibly inactivated functional subunits (i.e., replicons) of chromosomal DNA as was proposed (Fujita, 1965a).

It has been pointed out that the DNA portions that are irreversibly inactivated are characterized by four extraordinary features (Fujita, 1965a; Caplan and Ordahl, 1978; Goldman *et al.*, 1984): (i) Incapability of RNA synthesis, (ii) shortened and condensed structure even in the interphase, (iii) replication occurring late in the S-phase, and (iv) the acquired feature of the inactivated DNA inherited unchanged by the daughter cells through subsequent mitoses. This hypothesis was proposed almost 40 years ago but direct evidence to support it has been lacking until now. The results of the experiments of Caplan and Ordahl (1978), Goldman *et al.* (1984) and Takizawa *et al.* (2001), however, have provided strong evidence, though circumstantial, supporting the major differentiation hypothesis.

Those replicons that have escaped irreversible inactivation can be switched on and off according to extra- and

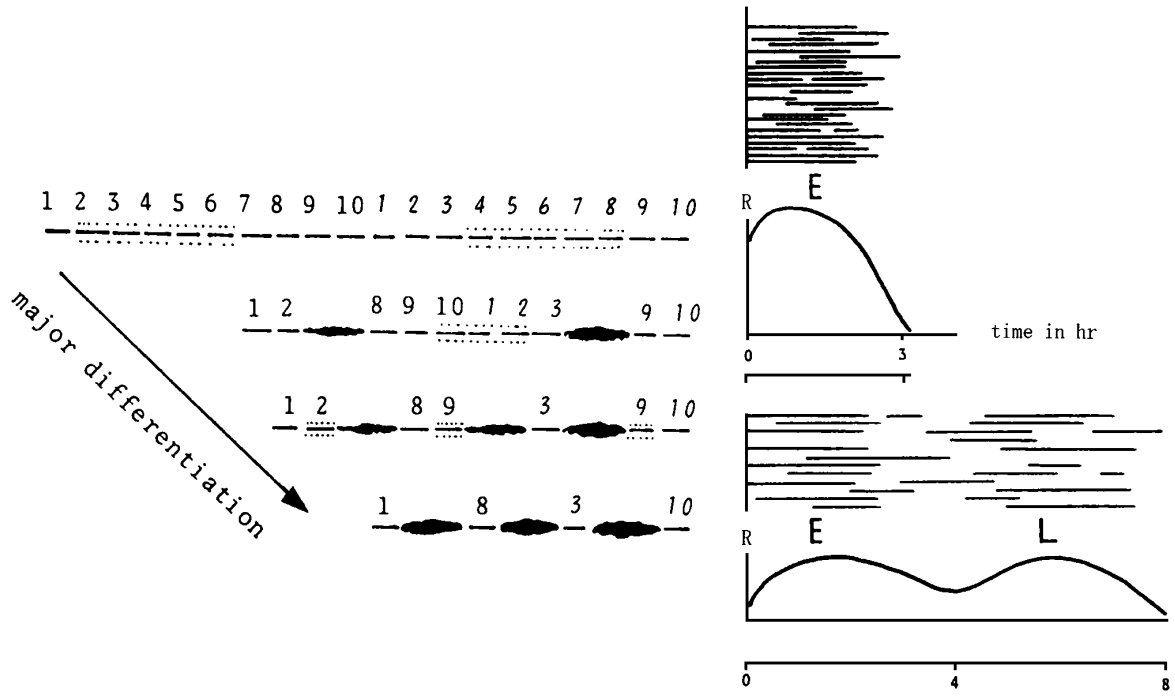


Fig. 17. Major differentiation: A model showing changes in chromosomal organization and DNA synthesis in relation to major differentiation. On the left side of the figure, chromosomal changes during development are illustrated. In an undifferentiated cell (top), all the replicons composing the chromosome are not irreversibly inactivated, although only a few of them are actively transcribing mRNA (dotted segments). All the replicons, as shown in the figure at the top right, are early replicating (E) and the rate of DNA synthesis (R) is expected to form a simple pulse-shaped curve. The absolute length of the S-phase should be short. While major differentiation proceeds, many replicons are irreversibly inactivated as shown in the condensed state in this diagram (left). They become late replicating (L) and make the curve of the rate of DNA synthesis complicated (right, below). The length of the S-phase also becomes longer with additional late replicating segments. The irreversible inactivation of genes is the genetic basis of determination of cell differentiation, and reversible on and off switching of potentially active genes corresponds to functional modulation of the cell. Adapted from Fujita,1986.

intracellular signals. This phenomenon corresponds to environmental modulation of genetic functions, which is designated as minor differentiation (Fujita, 1965a).

16. Major differentiation starts in matrix cells

In the analysis of the elevator movement of matrix cells in various species of animals, an unmistakable tendency of steady elongation of cell cycle and DNA synthetic times during development was found (Fujita, 1962; Hoshino *et al.*, 1973). This tendency has been observed in all animals so far studied. Besides matrix cells of neurectodermal origin, chick erythroblasts (Holtzer *et al.*, 1977), endodermal cells in the frog *Xenopus laevis*, cells of blastomeres of the sea urchin embryo (Dan *et al.*, 1980), the ectodermal cells of *Cynops pyrrhogaster* (Yamazaki-Yamamoto *et al.*, 1984) etc. have been reported to show the same tendency. According to the hypothesis of major differentiation (Fujita, 1965a), the length of the S-phase is expected to become longer in differentiated cells in comparison with that of their undifferentiated precursors, as illustrated in Figure 17.

In the beginning of the vertebrate ontogenesis, none of the replicons in the zygote are irreversibly inactivated: The cell is accordingly in its maximally totipotent state. DNA replicons in the cell begin to synthesize their DNA synchronously at the onset of the S-phase, at a uniform velocity so that the overall rate of DNA synthesis of the cell shows a simple pulse-shaped curve (Fig. 17, above right). The length of the S-phase is expected to be short. As the cell progresses by steps of major differentiation (Fig. 17, left), irreversibly inactivated replicons increase in number and the S-phase becomes longer (Fig. 17, below right). The curve of the overall rate of DNA synthesis is now expected to have multiple peaks as shown in the diagram in Figure 17 (right). The tendency toward steady elongation of the S-phase in matrix cells of developing vertebrate embryos (Fujita, 1962; Hoshino *et al.*, 1973) seems to support the notion that matrix cells steadily progress by steps of major differentiation as development proceeds.

17. Major differentiation directs the formation of neurons and glial cells

If one assumes the above hypothesis that irreversible inactivations of genes determine the fate of the cell while the matrix cells are actively proliferating, and that the type of cell differentiation is determined by a specific combination of irreversibly inactivated replicons, one can understand the characteristics of matrix cell differentiation and neuron production as follows (Fig. 18).

Although matrix cells keep their epithelial morphology unchanged from the very beginning of the neural plate formation to the end of stage II of cytogenesis, they change their state of major differentiation steadily as development proceeds. When they repeat mitoses and enter into G1 phase, irreversibly inactivated replicons increase in number and the cell accumulates the steps of major differentiation. The combinations of the inactivated replicons and their distribution patterns are supposed to be different in different cells: Two daughter cells born from the same matrix cell inherit the same pattern of inactivated replicons but can acquire new inactivations on different additional replicons forming different subclones in terms of major differentiation.

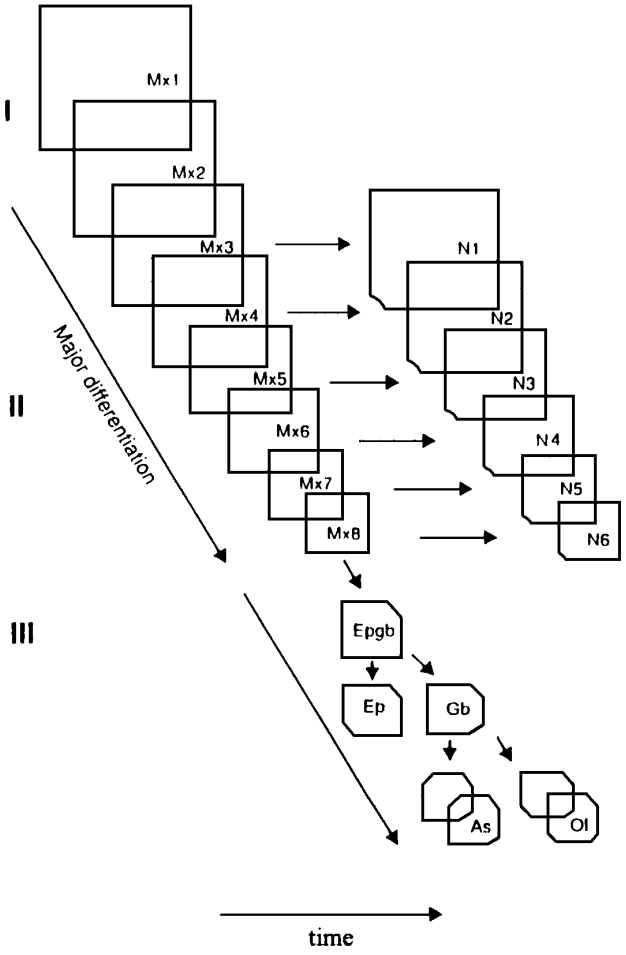


Fig. 18. Schematic diagram showing progression of irreversible differentiation (major differentiation) of matrix cells during development of the CNS. The state of major differentiation of matrix cell population is progressively changing (from Mxl to Mx8). From each state of major differentiation, specific neuroblasts (N l to N6) are produced. Their specific states of differentiation are predetermined by those of their immediate precursors, i.e., of the matrix cells. What determines the transition from matrix cells to neurons may be a common inactivation of one replicon that contains genes essential (either directly or indirectly) for DNA replication. Sizes and shapes of the frames of differentiating cells in this figure represent the potency of cells at their respective stages of major differentiation. I, II, III correspond to stages of cytogenesis. Abbreviations: Epgb, ependymoglioblast; Ep, ependymal cell; Gb, glioblast; As, astrocyte; Ol, oligodendrocyte. Adapted from Fujita, 1997.

Figure 18 depicts one branch of the matrix cell subclones. Frames Mxl to Mx8 represent the magnitudes of differentiation potencies of matrix cells at given stages of major differentiation. When major differentiation reaches a certain level, matrix cells can differentiate neuroblasts (commencement of stage II). Neuroblast differentiation in the vertebrate CNS is characterized by absolute repression of DNA replication; it is possible that neuroblast differentiation from matrix cells may be determined by an irreversible switching off of a gene or genes directly or indirectly related to DNA duplication for cell proliferation. It has been proposed (Fujita, 1986) that the differentiation of all neurons is commonly determined by one additional inactivation of this kind of replicon in the genome of the matrix cells at a certain state of major differentiation (Mxl through Mx8). If one can assume this mechanism, it is easily understood why highly specialized neurons in the CNS are produced at given times and places during stage II of cytogenesis, and why their principal fates are irreversibly fixed at their birth dates. If one assumes that the differentiation of neurons and neuroglia is determined by the irreversible repression of replicons during neurogenesis as discussed above, many important problems of cell differentiation in the CNS, such as the transition between stage II and stage III of cytogenesis can be explained in a simple way. Namely, if major differentiation of matrix cells progresses and the neuron-essential genes, without which no neuronal activity can be realized, are irreversibly inactivated in a matrix cell, it can no longer produce neurons. What the matrix cell can differentiate are nothing else but non-neuronal cells, i.e., neuroglial cells. Phenotypically, this signifies the beginning of stage III of cytogenesis. The progressive gene inactivation model or the major differentiation hypothesis would explain the sequential occurrence of stage I, II and III, and the production of specific cells in specific stages of the development of the vertebrate CNS. Not only would it explain the

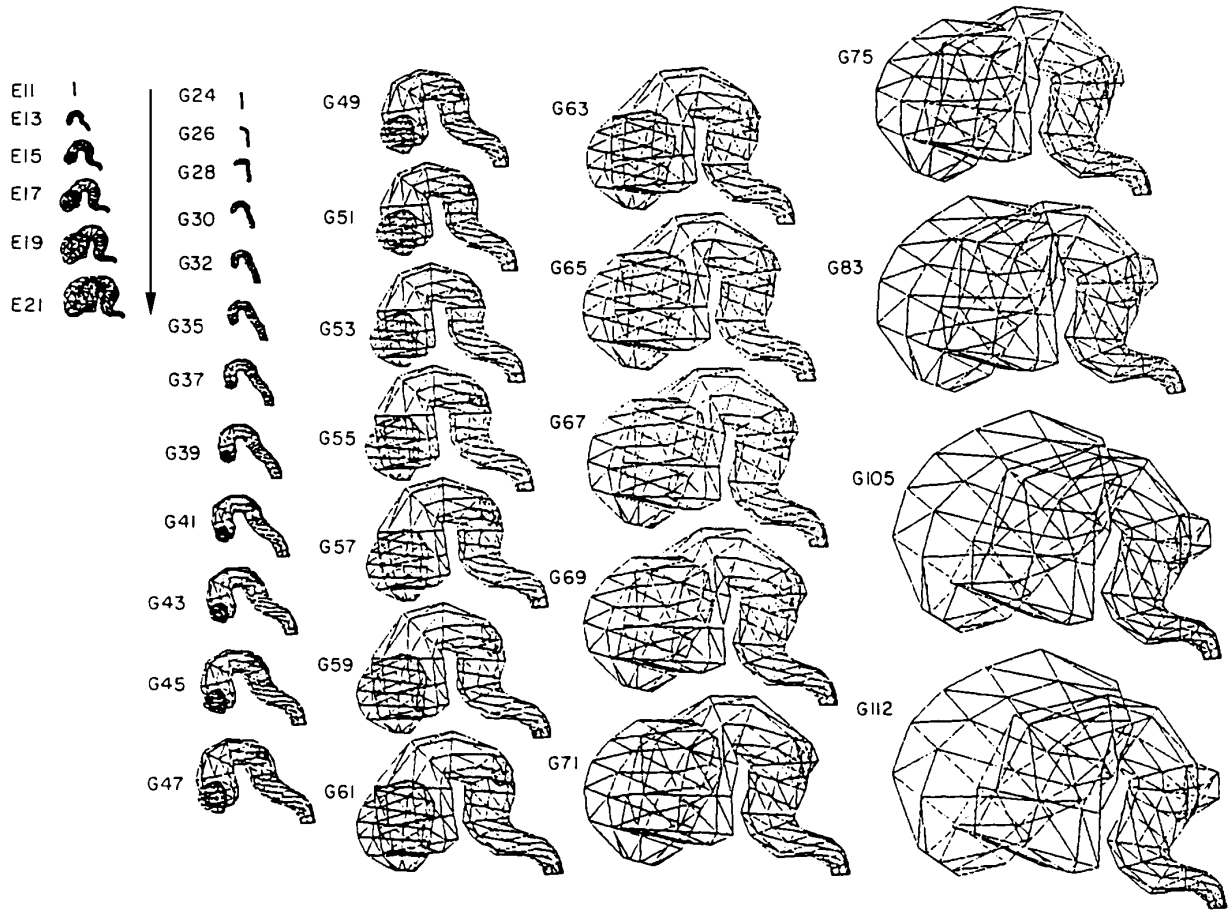


Fig. 19. Computer simulation of brain morphogenesis. Based on the matrix cell theory, differential growths in various regions of the brain are calculated by applying a single form of growth function (Gompertzian function) with different growth rates of matrix cells in different locations, and the morphogenesis of human brain can be reproduced in satisfactory fidelity in temporal and size scales. Changing the parameters, the same simulation technique can be applied to other species, as shown in the Figure (for example rat brain, at the left) (Fujita, 1990). E, embryonic days of rat fetus; G, gestational days of human fetus.

cytogenesis but also it would enable us to analyze genetic mechanisms of cellular differentiation in the developing CNS in molecular terms: When, where, and what kind of specific gene or genes are inactivated to determine the differentiation of various kinds of neurons or neuroglia, may be analyzed based on this hypothesis. An extremely complicated pattern of cell-differentiation in CNS, as it would appear at first glance, might turn out to be the result of simple hierarchical repressions of certain classes of essential genes. Some of these genes, of which GliaCellsMissing (Hosoya *et al.*, 1995) and NeuroD (Lee, 1997) seem to be remarkable examples, have been discovered recently.

18. Computer simulation of brain morphogenesis by the matrix cell

As I confirmed that the matrix cell population during stages I and II were essentially homogeneous so far as a narrow region was concerned, I could further develop computer simulation of brain morphogenesis, assuming the steady elongation of the cell cycles during stages I and II, as calculated from kinetic studies of the matrix cells, and differential growth rates in different regions of the brain.

Applying simple growth equations of the same form,

$$dW_i/dt=\delta_i(t)=\alpha_0 \cdot \exp(-\lambda_i t)$$

with different growth rates λ_i in different locations i of the neural tube, ontogenetic development of mouse, rat, monkey and human brains could be reproduced with satisfactory fidelity of time and size scales (Fig. 19) (Fujita, 1990).

19. Evo-devo of the human brain as viewed from the matrix cell theory

The same simulation technique can be applied to study the evolution of the human brain: Apparently so complex a structure as the human brain must have been developed as a result of a differential pattern of proliferation and differentiation of matrix cells in the neural tube during the long history of the evolution. A unified theory of brain evolution in morphological and functional aspects of changing ontogenesis from the standpoint of the matrix cell theory (Fujita, 1997) will no doubt be an important subject of future investigation.

20. Recent re-discovery of the matrix cell nature of the neural stem cells

Quite recently many investigators have come to a definitive conclusion on the stem cell nature of the so-called radial glia (Qian *et al.*, 2000; Miyata *et al.*, 2001; Noctor *et al.*, 2001; Parnavelas and Nadarajah, 2001; Tamamaki *et al.*, 2001; Temple, 2001; Nakamura, 2002, 2003; Barres, 2003; Morest and Silver, 2003). Their conclusions are virtually unanimous on the point that the so-called radial glia are not "glia" in the usual sense of the term but proliferative stem cells producing first neuroblasts and later glioblasts. Some of these authors rightly cast doubt about calling the multipotent stem cells by the name of "(radial) glia". These cells are no different in essential properties from the matrix cells as described over the last 45 years, and need not be called by the separate and misleading name of "glia".

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