Int. J. Dev. Biol. 38: 175-183 (1994)

Special Review

Early events in the histo- and cytogenesis of the vertebrate CNS

JUNNOSUKE NAKAI[1]* and SETSUYA FUJITA[2]

[1]*Hamamatsu Photonics, Hamamatsu and* [2]*Department of Pathology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

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***Address for reprints:** Hamamatsu Photonics, 4-26-25, Sanarudai, Hamamatsu 432, Japan.

0214-6282/94/$03.00

Printed in Spain

Prelude to neurogenesis

Neural induction has been regarded as the earliest event in neurogenesis in all the vertebrate embryo. Before neural competence appears, however, continuous cellular and chromosomal processes proceed in the cleaving embryo culminating in neural induction. In aplacental animals, such as amphibia, reptiles and birds, the first 10 or so cleavage divisions are mostly synchronous and the cell cycle is very short, virtually lacking G1 and G2 phases. Yamazaki-Yamamoto *et al.* (1980, 1984) have studied changes in C banding patterns and morphology of chromosomes in *Cynops pyrrhogaster* embryos and found that during this phase (from fertilization to the beginning of blastula stage), the metaphase chromosomes remain unchanged in morphology and C banding pattern. They found, however, that remarkable shortening, almost halving in length, occurred from blastula to gastrula stage accompanied by a prominent decrease in chromosome volume, as the chromosomal width was kept constant. During this period, many C bands fused with the neighboring ones and reduced in number as the blastula proceeded to neurula The neural competence appeared in blastula stage, immediately after the shortening of the chromosomes began. It is well known that at the same time, dermal differentiation in the embryo is determined and morphogenetic movements and migration of individual dermal cells begin to take place.

These chromosomal changes that seem to lead to neural induction and neurogenesis are characterized by the remarkable elongation of S-phase in the cell cycle, from 3 h in morula stage to 38 h or more in neurula stage (Yamazaki-Yamamoto *et al.*, 1980). The time spent for DNA replication is prolonged (Fig. 1). The same phenomenon continues in the neural tube. Autoradiographic studies have revealed remarkable increases in DNA-synthetic times during neural tube development as reported by Fujita (1962, 1986), Hoshino *et al.* (1973) and others. According to Hyodo and Flickinger (1973), the velocity of DNA replication within each replicon, as studied in frog embryos, is almost constant, being 6 μm/h. The length of each replicon, however, increases as the development of frog *Rana fusca* proceeds, being estimated at 11, 27 and 29 μm, in gastrula, neurula and tail bud stage, respectively. Obviously, initiation sites of DNA replication in some replicons are progressively obliterated or, in other words, many replicons fuse with the neighboring ones as development proceeds. The same phenomenon may be reflected in the observation mentioned above that many C bands fused with the neighboring ones and reduced in number as the blastula proceeded to neurula. The obliteration of initiation sites of DNA replication seems to be accompanied by the absolute incapability of RNA synthesis on that replicon, while DNA appears to be replicated as a continuation from the neighboring replicons though taking longer to complete the replication. At the light microscopic level, the synchrony of the cell division is lost rapidly during this period as a result of progressive elongation of the cell cycles.

Abbreviations used in this paper: CNS, central nervous system; GFAP, Glia fibrillary acidic protein; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Nature of matrix cells, pluripotent precursor cells in the CNS

The analysis of cell proliferation and differentiation of stem cells in developing vertebrate embryos revealed that there are highly regulated patterns in the genesis of neuronal and glial populations in the vertebrate CNS. At the beginning, there is a stage in which the neural tube is composed solely of pluripotent progenitor cells, matrix cells (matrix means "progenitor", Fujita, 1962). At this stage (called stage I), matrix cells proliferate only to multiply themselves (Fujita, 1963). They perform the elevator movement synchronous to their mitotic cycle (Fig. 2A).

The matrix cells compose pseudostratified columnar epithelium of the neural tube and adhere, during interphase, to each other with N-cadherin (Takeichi, 1990). When they enter into mitosis, the adhesion ceases and the cell bodies round up. Throughout mitosis, the junctional complex in the apical process remains unchanged so that the cell body is inevitably pulled towards the ventricular surface. Fujita (1990) examined distribution of F-actin that lines the cell membrane of the dividing matrix cell by confocal microscopy and concluded that F-actin is depolymerized during mitosis, released from linker molecules binding cadherin and F-actin and, as a result, dissociation of the cadherin-mediated adhesion takes place during mitosis (Fig. 3B). Fujita (1990) regards this cyclic modulation of N-cadherin adhesion during mitosis as the mechanism of the elevator movement.

After several mitoses during stage I, matrix cells begin to differentiate neuroblast. This stage of neuron production is called stage II of cytogenesis (Fujita, 1964). There appears to exist a rigid and close correlation between the time and place of birth and the type of neuron differentiation determined at the birth of each neuroblast.

Once the neuroblast is differentiated from matrix cells, most, if not all, features of the future neuron are irreversibly fixed and cannot be altered by subsequent dislocation or environmental changes. In the CNS, modification of neuron types can only occur at an early stage of matrix cells if the environment is changed. This has been confirmed (Cavines, 1982) in the mutant mice in which location of the cortical neurons is drastically disordered but type of

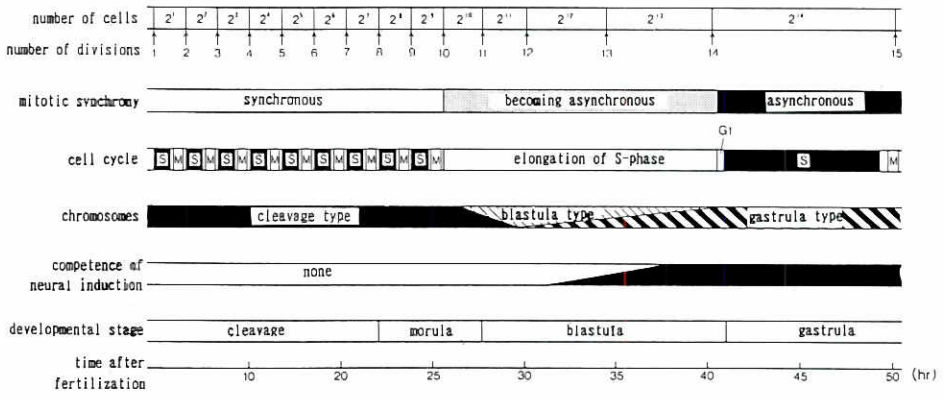

Fig. 1. Cell division, chromosomal changes and appearance of neural competence in early development of *Cynops pyrrhogaster* *(Yamazaki-Yamamoto et al., 1980). They designated short and condensed chromosomes at gastrula stage as "gastrula type chromosomes".*

neuron differentiation unchanged. Transplantation into heterotopic sites (Nakamura *et al.*, 1986) or explantation *in vitro* have also failed to change the original type of differentiation of neurons in the CNS. It is likely that determination of the neuroblast differentiation takes place at an early G1 of the matrix cell (Fujita, 1963).

When all the neurons are produced, stage II ends and the stage of neuroglia production begins (Fujita, 1965b). Matrix cells are now restricted to producing only non-neuronal cells and change into ependymoglioblasts, which are soon differentiated into ependymal cells and glioblasts. This is stage III of cytogenesis. The glioblast (Fujita, 1965b) is a common progenitor of neuroglial cells of the CNS, and first differentiate astrocytes, then oligodendroglia and finally metamorphose into the microglia (Fujita *et al.*, 1981). Based on the monophyletic theory described above, cell differentiation in the developing vertebrate CNS is explained with a simple scheme shown in Fig. 3A.

GFAP and matrix cells

Recently, however, several authors (Antanitus *et al.*, 1976; Levitt *et al.*, 1983; Choi, 1986) have reported that they could observe a strong reaction of antiGFAP antisera in matrix cells at early stages of development, and claimed that neuroglial differentiation proceeds parallel with the production of the neuroblast (Fig. 3B). If matrix cells produce such a great amount of GFAP, their relationship to neuroglial differentiation would have to be re-examined.

Those investigators who have observed the positive GFAP reaction have used antisera provided by Eng or Bignami and Dahl (Antanitus *et al.*, 1976; Levitt *et al.*, 1983; Choi, 1986). Curiously enough, Eng or Bignmi and Dahl have never found any positive GFAP reaction in matrix cells at stages I and II with their own antisera. Critical examination is obviously necessary.

We have investigated this problem using chicken, mouse, rat, bovine and human fetal brains and spinal cords by applying immunohistochemical staining, chemical analysis with SDS PAGE and immuno-blotting (Fujita, 1986), and came to the conclusion that GFAP is not present as protein in stage I and II matrix cells at least, at detectable level by present day techniques. This conclusion, however, is not definitive, since a possibility still remains that the signal of the GFAP might be transcribed but not translated during stage II of cytogenesis.

In order to examine when and where the gene of GFAP is transcribed, we prepared cDNA from mRNA of fetal bovine spinal cord (Fujita *et al.*, 1986). The occurrence of the GFAP-encoded mRNA is studied using this cDNA probe. The signal of GFAP-encoded mRNA is found only postnatally, and no trace of the signal is detected in RNA fraction of fetal rat cerebrum (embryonic day 16, 22). A similar observation was reported by Lewis and Cowan (1985) with mouse GFAP-mRNA. They found weak positive signal first at postnatal day 2, which increases until day 9 followed by a slight decrease to the adult. In their experiment, embryonic mouse brains did not show any positive signal of GFAP-encoded mRNA. In chicken brains, Capetanaki *et al.* (1984) also obtained the same result, i.e., that up to day 10 of incubation (the end of stage II) no trace of GFAP mRNA was detectable by Northern blotting but that strongly positive signal appeared in the spinal cord 2 weeks after hatching.

The result reveals that the occurrence of the GFAP-encoded mRNA parallels the appearance of the GFAP molecules in the developing CNS as described previously (Fujita, 1986), and that, in rat as well as chicken and mouse brains, transcription of GFAP-encoded gene first occurs some time after stage III of cytogenesis commences, but not concomitant with neuroblast production.

Applying Northern blotting, GFAP-encoded mRNA is found to appear first at stage III of cytogenesis. It is, however, not clear in what cells it occurs and what distribution in space it takes. We studied this problem using *in situ* hybridization to examine how GFAP-encoded mRNA is transcribed during development of the rat brain. At embryonic days 16 and 22 no positive cells were detectable in the cerebral hemisphere, corresponding to the result of immunohistochemistry of GFA protein. First postnatally positive cells appears in subependymal position at day 4 and subsequently increased in number between days 12 and 23. These cells were immature neuroglial cells and could be identified as astrocytic cells (Fujita *et al.*, 1986). Summarizing these observations at protein and mRNA levels, we conclude that GFAP is not present in stage I or stage II of cytogenesis and it is right to conclude that the classical concept of "primitive spongioblasts" (cf. Fig. 3B) or "radial glia" as committed precursors of neuroglia that are claimed to be present in an early phase of cytogenesis of the vertebrate CNS is not tenable.

Determination of cell differentiation and its possible genetic mechanism

It is now generally believed that development of the vertebrate, when viewed at the cellular level, proceeds by sequential steps in

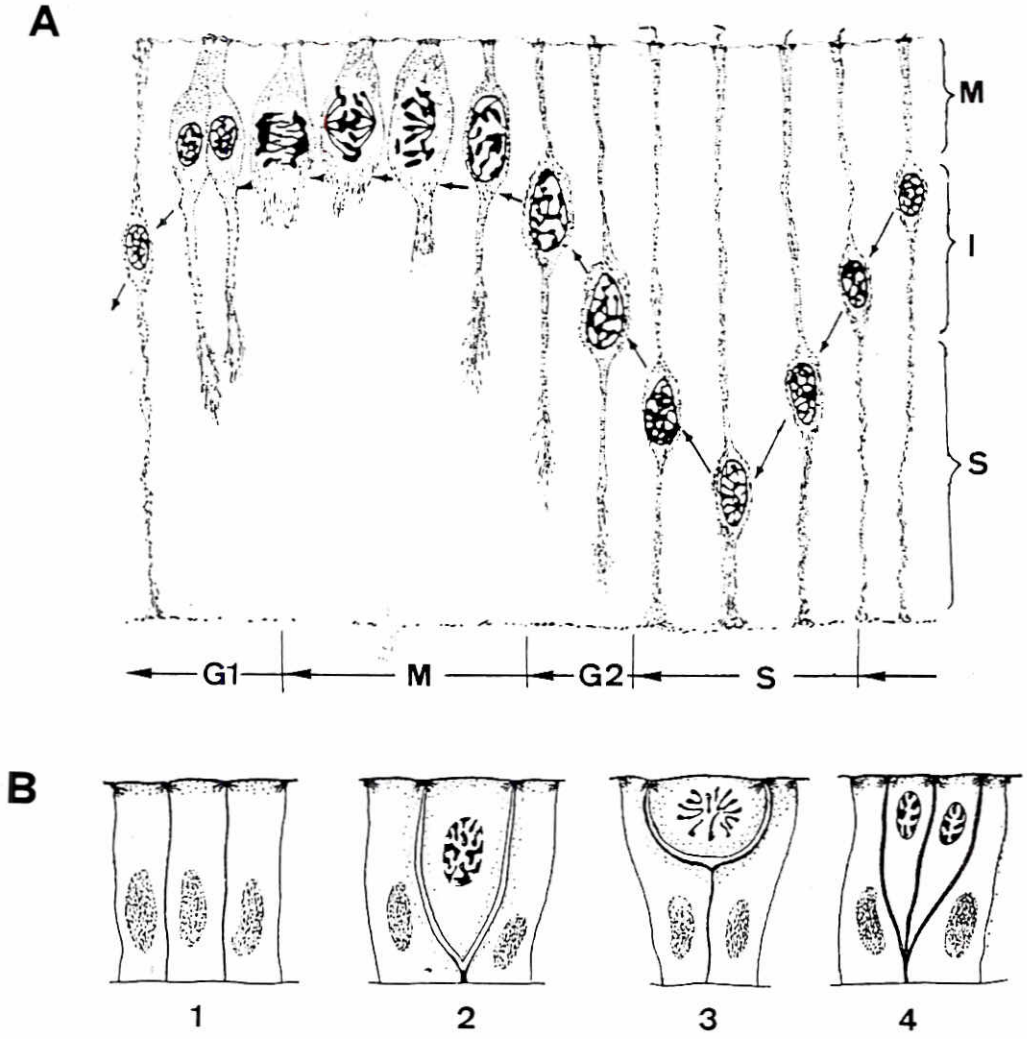

Fig. 2. Elevator movement of matrix cells. (A) *Matrix cells perform an elevator movement synchronous with their mitotic cycle (adapted from Fujita, 1962).* **(B)** *The mechanism of the movement is supposed to be due to cyclic changes of cell adhesion to neighboring cells. Linker proteins and F-actin bound to N-cadherin are depolymerized during mitosis and, as a result, N-cadherin loses its homophilic adhesion ability (Fujita, 1990).*

which the potencies of progenitor cells become progressively and irreversibly restricted. This type of differentiation has been called "major differentiation" (Fujita, 1965a), in contrast to reversible expression and repression of genes, or "minor differentiation". The simplest hypothesis to explain the mechanism of the restriction of the potencies is to assume progressive and irreversible inactivations accumulating among functional subunits (i.e., replicons) of chromosomal DNA (Fig. 4). It has been pointed out that the DNA portions that are irreversibly inactivated are characterized by 4 extraordinary features (Fujita, 1965a; Caplan and Ordahl, 1978; Goldman *et al.*, 1984):

1) incapability of RNA synthesis,
2) shortened and condensed even in the interphase
3) replicating late in the S-phase, and
4) the acquired feature of the inactivated DNA is inherited by the daughter cells unchanged through subsequent mitoses.

Although it was almost 30 years ago that this hypothesis was proposed, direct evidence to support this hypothesis of "the major differentiation" has long been lacking. The recent introduction of analytical techniques of molecular biology has made it possible to test the hypothesis by using cDNA probes of specific genes in specific chromosome sites and identifying the timing of their replication in the S-phase of various types of cells at differentiated and undifferentiated states (Epner *et al.*, 1981; Furst *et al.*, 1981; Goldman *et al.*, 1984). This result of the test now provides strong evidence to support the hypothesis.

Major differentiation of matrix cells

In analyzing of the elevator movement of matrix cells in various species of animals (Fujita, 1962, 1986), an unmistakable tendency of steady elongation of cell cycle and DNA synthetic times during development was found. This tendency has been observed in all the animals in development so far studied. Besides matrix cell of neurectodermal origin, erythroblasts of chicken (Holtzer *et al.*, 1977), endodermal cells in *Xenopus laevis* (Graham and Morgan, 1966), cells of blastomeres of sea urchin embryo (Dan *et al.*, 1980), etc. have been reported to show the same tendency. Ectodermal cells of *Cynops pyrrhogaster* (Yamazaki-Yamamoto *et al.*, 1984) described in the preceding section is one typical example.

According to the hypothesis of "major differentiation" (Fujita,

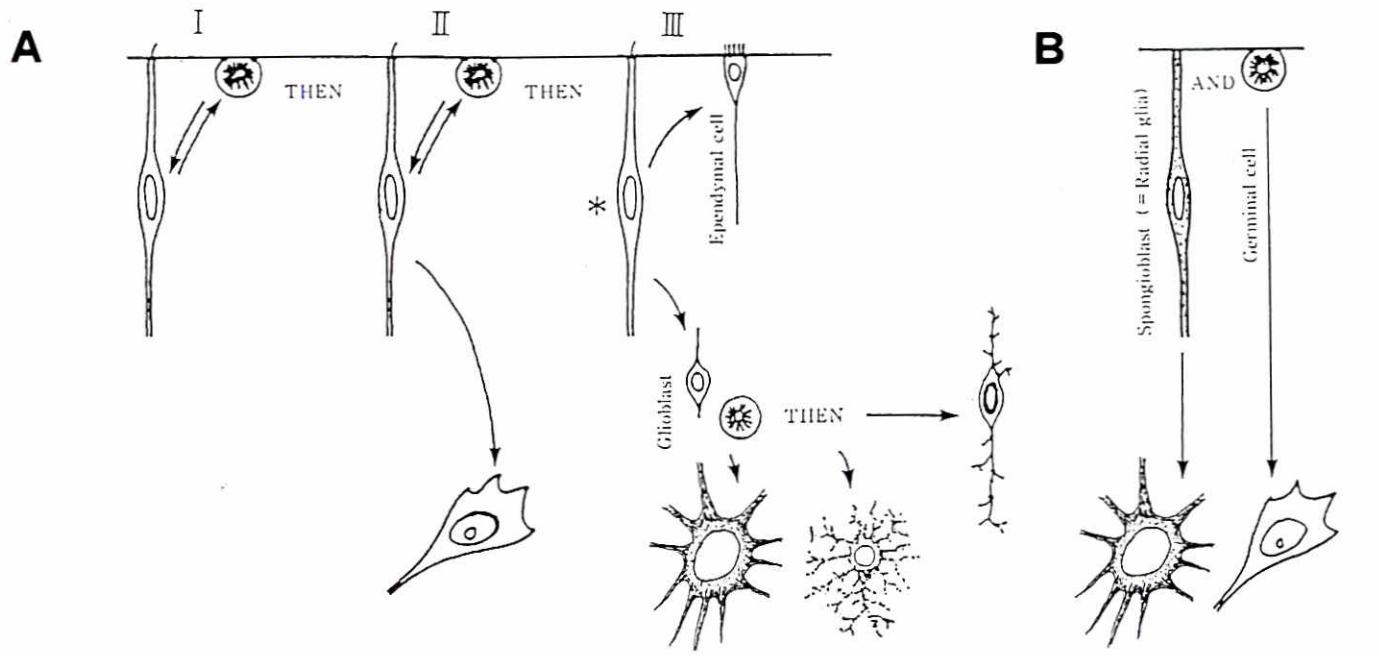

Fig. 3. Two theories of cytogenesis of the vertebrate CNS. (A) *Cell proliferation and differentiation proceed through 3 consecutive stages (Fujita, 1963, 1964). In stage I, the neural tube is composed solely of matrix cells, which perform an elevator movement ( ). In stage II, matrix cells give rise to neuroblasts in preprogrammed order. When all the neurons are produced, matrix cells change into ependymoglioblasts (*), common progenitors of ependyma and neuroglia. This is the beginning of stage III of cytogenesis. The ependymoglioblasts are rapidly differentiated into ependymal cells and glioblasts. The latter give rise to astocyte, oligodendroglia, in sequence, and finally metamorphose themselves into microglia (Fujita et al., 1981).* **(B)** *Rakic's radial glia theory or His' germinal cell theory. Rakic (Levitt et al., 1983) distinguished two committed stem cells in the wall of the neural tube: elongated radial glia (Spongioblast of His) and rounded germinal (or ventricular) cells. He believed that the former were specialized glial precursors and the latter, neuron precursors. Recently Levitt et al. (1983) found that many elongated cells in the embryonic brain reacted strongly with antiGFAP antisera, and concluded that His' spongioblasts are nothing but the radial glia co-existing with neuron-producing ventricular cells. The sole evidence on which they depend, however, is the positive reaction (dots in Fig. 1B) of antiGFAP antisera in the cells composing embryonic brain vesicles.*

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 Fig. 4.

At the beginning of the vertebrate ontogenesis, none of the replicons in the zygote are irreversibly inactivated, corresponding to its totipotent state in the major differentiation. 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. 4, left). The length of the S-phase is expected to be short. When the cell progresses in steps of the major differentiation, i.e., as the cell is differentiated, irreversibly inactivated replicons increase in number so that the S-phase becomes longer. The curve of the overall rate of DNA synthesis of the cell is now expected to have multiple peaks

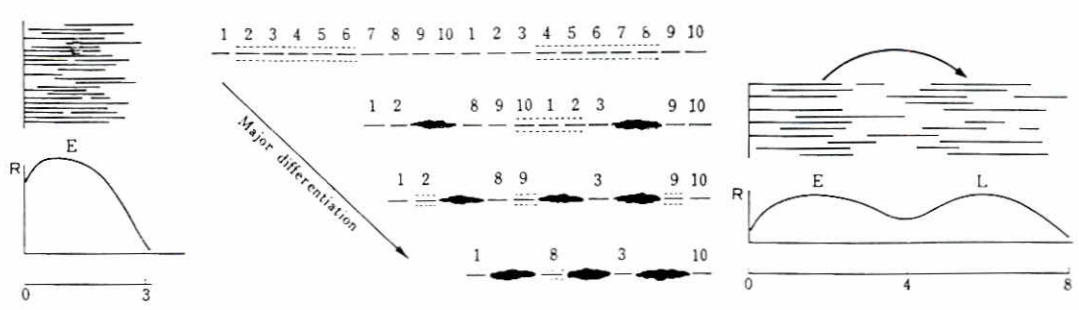

Fig. 4. Changes in chromosomes and DNA synthesis in relation to major differentiation (Fujita, 1965a). *In the center, chromosomal changes during development are illustrated. In an undifferentiated cell (top), replicons composing the chromosome are not irreversibly inactivated, although only a few of them are actively transcribing mRNA (dotted segments). All replicons as shown in the figure at the left 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 (center). They become late replicating (L) and make the curve of the rate of DNA synthesis complicated (right figure). The length of the S-phase also becomes longer with additional later replicating segments. The irreversible inactivation of genes is the genetic basis of determination of cell differentiation, and reversible on-off-switching of potentially active genes corresponds to functional modulation (minor differentiation) of the cell (Fujita, 1965a).*

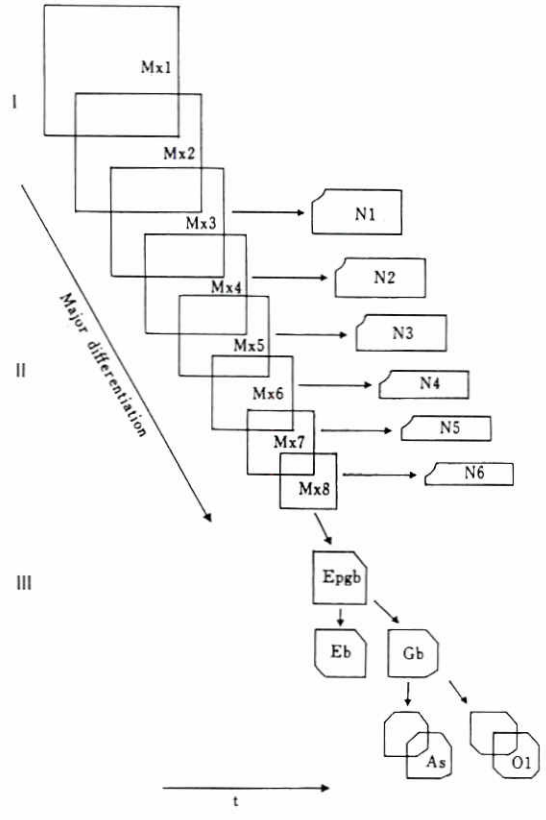

Fig. 5. Schematic diagram showing progression of irreversible differentiation (major differentiation) of matrix cells during development of the CNS *(Fujita 1975). The state of major differentiation of matrix cell population is progressively changing (from Mx1 to Mx8). From each stage of major differentiation, specific neuroblasts (N1 to N6) are produced. Their specific states of differentiation are predetermined by those of their immediate precursors, i.e., of the matrix cells. What determined the transition from matrix cells to neuroblasts may be a common inactivation of one replicon that contains genes essential for DNA replication. Sizes and shapes of the frames of differentiating cells in this figure represent potency of cells at respective stages of major differentiation. I, II, III correspond to stages of cytogenesis; Epgb, ependymoglioblast; Eb, ependymoblast; Gb, glioblast; As, astrocyte; Ol, oligodendroglia.*

as shown in the right-hand diagram of Fig. 4 and the S-phase becomes longer.

The tendency toward steady elongation of the S-phase in matrix cells of developing vertebrate embryos (Fujita, 1962, 1986) seems to support the notion that matrix cells steadily progress in steps of the major differentiation as development proceeds.

Major differentiation and formation of neuronal and neuroglial cells

If we assume the above hypothesis that irreversible inactivations of genes determine differentiation of the cell even when the stem cells are actively proliferating, and that the type of cell differentiation is determined by the specific combination of the irreversibly inactivated replicons, we can understand the characteristics of matrix cell differentiation and neuroblast production as follows.

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 cells accumulate 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 the inactivated replicons but can acquire new inactivations on different additional replicons forming different subclones in terms of major differentiation. Figure 5 shows one branch of the matrix cell subclones.

Frames of Mx1 to Mx8 represent the magnitude of differentiation potencies of the matrix cells at respective stages of the major differentiation. When the 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 cell 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, 1975) that the differentiation of all neuroblasts (or 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 (Mx1-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 their future fates are irreversibly fixed at the time of birth.

If we can assume 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 transition of stage II of cytogenesis into stage III, can be explained in a simple way. Namely, if major differentiation of matrix cells progresses and 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 they can differentiate are nothing but non-neuronal cells, i.e., neuroglial cells. Phenotypically, this signifies the beginning of stage III of cytogenesis.

The progressive gene inactivation hypothesis or the major differentiation hypothesis can explain nicely the sequential occurrence of stages I, II and III and production of specific cells in the development of the vertebrate CNS. Not only can it explain cytogenesis, but also it enables us to analyze the genetic mechanism 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 kind of neurons or neuroglia may be analyzed by this technique. What at first glance appear to be extremely complicated patterns of cell differentiation in CNS might turn out to be the result of simple hierarchical repressions of certain classes of essential genes.

Irreversible gene inactivation and neural plasticity

We have proposed the hypothesis that cellular differentiation in the CNS is realized by progressive and irreversible inactivations of replicons that accumulate during ontogeny. According to this hypothesis, cells in the CNS are characterized by very rigid cytodifferentiation. Paradoxically, it is well known that the vertebrate CNS, particularly of the primate, is highly plastic at least in functional aspects. How the neural plasticity is related to the

irreversible differentiation of the cells of the CNS is explained as follows.

According to the hypothesis, the major differentiation of a cell is determined by the irreversible inactivation of some replicons. The inactivated replicons are supposed to be small in number among all the replicons of the genome. The rest, or majority of replicons, are in a potentially active state, and can be switched on and off in response to the signals of intra- and extracellular environment. This type of reversible expression of cellular differentiation, which has been called "minor differentiation", is characterized by reversible synthesis of mRNA and protein, and is a genetic basis of neural plasticity.

Mechanism of pathfinding and the principle of multiple assurance in neurogenesis

Pathfinding is, undoubtedly, one of the most important processes in neurogenesis. Historically, our study of pathfinding commenced with direct observation on living neurons in *vitro* that extended the axon to navigate in artificial media or cellular environment (Nakai and Kawasaki, 1959). Various media or environmental factors, e.g., coating the surface of culture chamber or scattered as minute particles on the substrate, were tested. As for the effect of the cellular environment to neuronal pathfinding and to formation of the connection, interactive behaviors of growth cones against other neurons, neuroglia, Schwann cells and skeletal muscles was studied (Nakai, 1979). The growing axons navigating *in vitro* showed characteristic movement of extension and retraction. Frequently the retracting axon pulled and dislocated the soma of neuron towards the place where the axon is attached. This extension and retraction of axon represents positive and negative strategies to establish final connection to the target.

The same strategy also characterizes movements of filopodia that extend and retract from growth cones (Fig. 6A). The velocity of the extension and retraction is high, both ranging from 1 to 17 μm/min and the distance of the extension is over 10 to 20 μm (Nakai, 1979). An interesting finding is that the filopodia not only move to and fro but swing around the fulcrum when they have free space to do so. Their angular velocity varied from 50 to 1,800 degree/min (Fig.6).

Obviously, the movement of the filopodia facilitates accomplishing molecular recognition on target cell surfaces by the growth cones when they approach the targets. Fujisawa *et al.* (1989) has found and characterized a membrane protein called A5, composed of 927 aminoacid residues. This molecule is expressed on the surface of axons of the optic ganglion cells as well as on cell membrane of the target neurons with which the retinal ganglion cells establish connection. Obviously this type of molecule plays a decisive role in establishing the specific connection, in this case, of visual neuron network. Many kinds of adhesion molecules are reported to play similar roles.

Before the growth cones draw near the targets, however, the axon must navigate towards the final objects. How this is accomplished is the problem. To understand this process observations *in vivo* are more important. Nakai (1975a) examined outgrowing axons of spinal motor neurons in chick embryos by a newly developed Bodian-Nomarski technique and found: (1) around stages 17-18 when the axons of motor neurons forming ventral nerve fibers begin to emerge, the sclerotomes also begin to develop segmentally from the ventral perichordal tissue growing

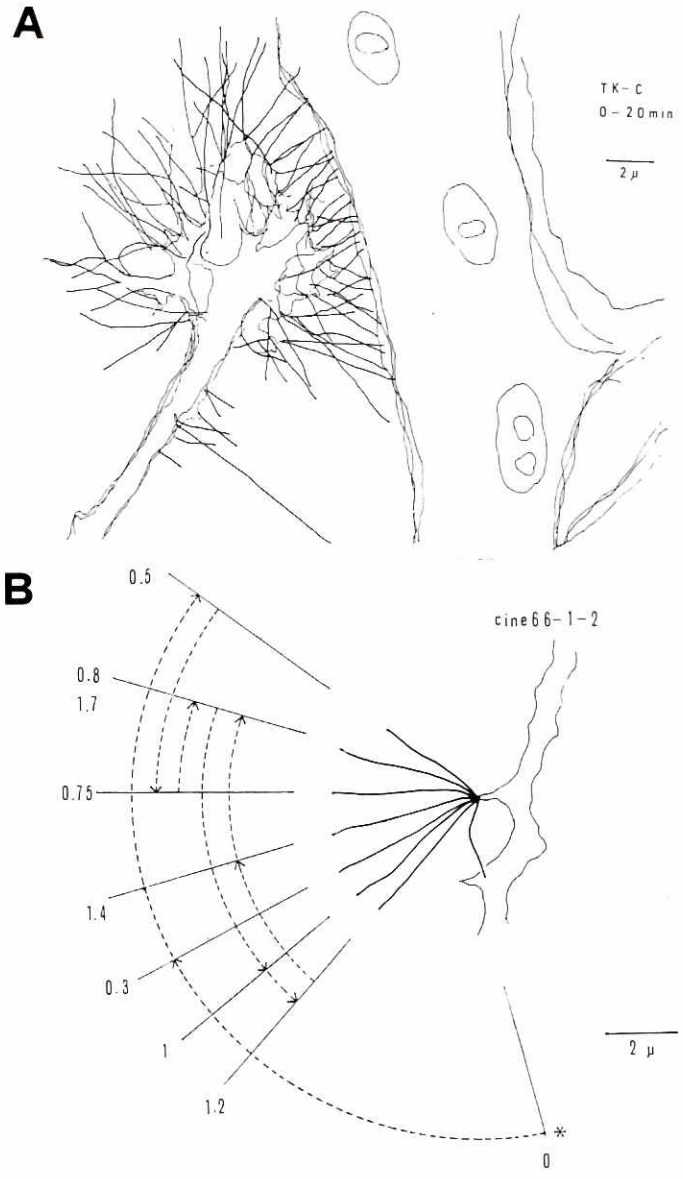

Fig. 6. Movement of filopodia (Nakai, 1979). (A) *Superimposed trace shows filopodia of a spinal ganglion cell sprouting against a skeletal muscle fiber, recorded in cinema film frames, during a period of 20 min.* **(B)** *Swinging movement of a filopodia. Motion starts at *, 0 min, and moves in the direction of the first arrow taking 0.3 min, then to the next, after 0.5 min, and so on.*

dorsal apposing the neural tube (Fig. 7). This part of the sclerotome is called the neural arch. The sclerotome at this stage is a solid structure that prevents penetration of growth cones or even filopodia, thus dividing the lateral surface of the spinal cord into segmented channels. Axons of ventral as well as dorsal root are inevitably divided into segmental channels;

(2) Exactly at this stage of development, the myotome of each somatic segment is differentiated and is located very close to the tip of pioneer growing axons in the spinal nerve. The distance is approximately 20-30 μm. The distance the filopodia can reach is up to 20 μm and the growth cone advances at a speed of 1 μm/h. Thus

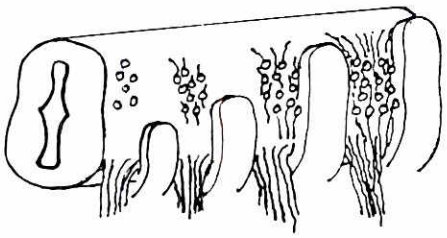

Fig. 7. Schematic drawing of correlation of development between neural arches and spinal nerves. *Development proceeds from left to right. (Nakai, 1975b).*

the growth cone of pioneering spinal motor neurons appears to be almost capable of directly reaching the target cells via filopodia immediately after emerging from the spinal cord and recognizes the surface molecule of the target, myoblasts. (3) Even when mesenchyme of 2-3 cell layers intervenes between the nerve tips and the target myoblasts, forming an obstacle for the filopodia, the growth cone itself traverses this distance within 20-30 min. Nakai (1975b) interpreted these 3 features as the triple assurance to the pathfinding of the spinal neurons. This situation seems not to be a particular case of the spinal nerve but rather a general phenomenon in developmental biology.

During the very long course of evolution, over 500,000,000 years since the creation of the vertebrate central nervous system, genetic changes that collaborate with pre-existing neurogenesis and providing additional assurance must have been preferentially fixed and have been accumulating to the present day, since this would increase survival value. Thus, it is no wonder that the complicated neurogenesis is supported by multiple assurance of important steps with which billions of neurons and neuroglia are assembled to perform the marvelous tasks of the brain in each individual of all the species of animals. This phenomenon, which can be observed everywhere in neurogenetic processes may be called the «principle of the multiple assurance» in developmental biology.

Summary

Development of the vertebrate, viewed on the cellular level, proceeds by sequential steps in which potencies of progenitor cells become progressively and irreversibly restricted. This is known as progression of the major differentiation. Cytogenesis of the CNS may be regarded as one typical example. The period of cytogenesis in the CNS is divided into three consecutive stages. In stage I, the wall of the neural tube is composed solely of matrix cells. In stage II, i.e., the stage of neuronogenesis, some of the daughter matrix cells are determined at the early G1 phase to be differentiated into neuroblasts. The specificity of individual neurons appears to be irreversibly determined at the time of birth of the neuroblasts, as a function of time-and-place of their production. The individual matrix cells that have existed at the very beginning of neurogenesis give birth to a series of progressively different types of neurons in stage II as the major differentiation proceeds. Finally, matrix cells cease to produce neurons. This is the end of stage II. Thereafter, only non-neuronal cells, namely neuroglia and ependymal cells, are produced. This is stage III or the stage of neuroglia production.

The sequential nature of the differentiative behavior of matrix cells can be explained by the hypothesis of progressive gene inactivations that accumulate in genomes of matrix cells during development. Different types of neurons are produced from matrix cells at different states of the «major differentiation». Irreversible inactivation of genes progresses during stage II of cytogenesis, and when genes essential for neuronal differentiation are inactivated in matrix cell genome, the cell can no longer differentiate neurons but necessarily produces only non-neuronal elements, i.e., neuroglia and ependymal cells. Thus the consecutive occurrence of stages I,II and III can be understood.

Studying pathfinding of spinal neurons, we found that the dynamic behavior of filopodia plays an essential role in guiding and establishing connection to the target cells. By extension, retraction and swinging movements, the filopodia can search for positive cues over an area of 40 μm in diameter. Examining how spinal nerves emerge to find out their target muscle cells in chick embryos, we found that (1) the developmental environment provides an adequate channel between neural arches to the target at the time the neuronis just beginning to send out its axon, (2) the distance between axon tips and the target is short when the axion first grows out. It is almost within a shooting range of the filopodia, and (3) if the filopodia can not directly touch the target cells, the growth cone proceeds and reaches the target within 30 min. These three features can be regarded as triple assurance for perfect pathfinding. Specific surface molecules to assure specific adhesion provide also the fourth assurance. Multiple assurance seems to be a general principle of development for the infallible assembly of morphological and functional organization in a developing CNS.

KEY WORDS: *vertebrate CNS, matrix cell, progenitor, neurogenesis, gliogenesis, genetic mechanism, pathfinding, filopodia, multiple assurance.*

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