FORMATION OF CELL MASSES IN THE MYELENCEPHALON OF THE CLAWED FROG, *XENOPUS MUELLERI*

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ABSTRACT

An important process in the organization of developing nervous system is the clustering of neurons with similar properties to form nuclei. The development of myelencephalon of Xenopus muelleri, a pipid frog that retains a lateral line system throughout life, was studied in Nissl stained serial sections. The results showed that density of neurons increases as the animal develops. Cell masses were formed in the latter half of larval stages (stage 48 to 59). Large neurons migrate first before small neurons. Raphes and reticular formation nuclei and Mauthner cells were the earliest neurons that could be distinctly recognized on the ventral part of the myelencephalon. By stage 54 out of 66 stages, the structure of the myelencephalon resembled that of the adult frogs.

INTRODUCTION

The course of neural development can be traced through four overlapping processes; cell birth (neurogenesis), migration, formation of connectivity (including elaboration of processes, synapse formation, cell death, and axonal regression), and myelination. The time when specific neurons are born can be detected using 3H-thymidine autoradiography (Altman and Bayer 1980). These studies in which labelled thymidine is permanently incorporated in DNA of dividing cells, have shown several principles of neurogenesis.

In a given region, there is characteristic "inside-out" pattern of origin, *i.e*., neurons that are originate later migrate past those born earlier to attain the most peripheral location. Large neurons are produced before small neurons, motor nuclei originate before sensory nuclei (at the same level of neuraxis) (Nadarajah and Parnavelas 2002).While a number of studies have been undertaken to explain cell masses of brain in adult frogs (Opdam *et al.* 1976, Matesz and Szekely 1978, ten Donkelaar *et al.* 1981, Nikundiwe and Nieuwenhuys 1983, ten Donkelaar 1998), the present study aimed at investigating the development of the myelencephalon from neurogenesis through the definite appearance of recognizable cell masses (nuclei) and secondly demonstrated how the observed pattern of development complies with the known principles of neurogenesis and neuron migration.

MATERIALS AND METHODS

Collection and Housing of Adult Frogs

Adult *Xenopus muelleri,* were obtained from the ponds using seine net and reared at normal room temperature (25 °C). The frogs were acclimatized in the laboratory for five weeks before they were allowed to breed. They were fed beef liver cut into small block twice a week and aquarium water was replaced before feeding. The aquaria were maintained at a diurnal light: dark cycle of approximately 12:12 hours, which is natural equatorial cycle.

Breeding

Spawning was induced by subcutaneous injection of Human Chorionic Gonadothrophin Hormone; Pregnyl (Organon, Oss, Netherlands) into the thigh. For a mating pair the male was given a daily injection of 300 I.U. for two consecutive

days, while the female was administered 100 I.U as a primer dose and 500 I.U five hours later (on the second day only). The mating pair was placed in a different darkened breeding aquarium with twigs for oviposition and left undisturbed until mating was over.

Care, Rearing and Staging of Tadpoles

Eggs were hatched within three days after mating, and feeding started on the fifth day. Tadpoles were fed with powdered *Amaranthus sp*. "**mchicha**" daily and water was changed just before feeding. After metamorphosis, animals were fed with *Lumbricus* (earthworms) and later they were switched to beef liver blocks. The developmental stages of the tadpoles were determined using standard tale of Nieuwekoop and Faber (1956).

Histological Techniques

Perfusion and Fixation of the Animals

To investigate the development of the myelencephalon, tadpoles of various stages were selected and anesthetized in a 0.025% solution of MS.222 (Sandoz). During processing the brains of stages 22 up to 44, the whole animals were fixed in 3% glutaraldehyde, embedded in epoxy resin, sectioned by an ultramicrotome (Reichert OmU3) at a thickness of 2 µm and stained with Toluidine blue O. From stage 45 up to 59, whole heads were taken and fixed directly in 10% formalin and then fixed secondarily in Boun´s fluid for paraffin wax embedding technique. Tissues were stained with cresyl violet for Nissl substances. Size of cells was obtained by averaging the diameters of their somata which were measured in two directions perpendicular to each other. The calibration was made at magnification of X 200 where the multiplying factor was 4.84 approximately 5 µm for each one unit of the eyepiece graticule. Photomicrographs of sections were made by a digital camera (FinePix 28000zoom model).

RESULTS

In the Nissl stained sections and in semi thin (2 µm) epoxy sections, growth, differentiation and migration of the neurons were observed.

Stage 22: At this early stage of development, neurons (N) in the myelencephalon were darkly stained, oblong in shape with an average diameter of (9.7 µm). They were distributed throughout its mass and attached to the dense meshwork processes, which are radially oriented from the ependymal layer in the ventricular zone to the peripheral area. These cells seem to be migrating in these processes. The whole structure of myelencephalon which, is normally observed at the level of otic vesicle (OT), was covered by an epidermis layer (EP). An embryonic brain cavity, the neurocoel (Ne) could also be observed (Fig. 1A).

Stage 25: At this stage, the myelencephalon anlagen was larger than in stage 22 tadpoles with the broader neurocoel, causing the lateral lips to bulge out more. Neurons occupy the whole of this area connected in the meshwork of processes like in the previous stage but with many vacuoles (V). The density of neurons had increased which probably imply that new cells were still being produced. The shape and size of individual neurons was similar to those of stage 22 (Fig. 1B).

Stage 33: There was a notable difference between this stage and the previous ones. The gross external shape of the myelencephalon anlagen consisted of a very thin roof and very thick lateral parts with a fourth ventricle (4^{th}V) in-between. This thin and delicate rhombencephalic roof was intact and consisted of a single-celled layer of ependymal (E). Neurons were still connected to a meshwork processes but the latter was not as conspicuous as in the previous stages. Some neurons, especially those in the ependymal area were darker and

smaller in size than those at the ventro-lateral parts. In the ventral parts, some of the neurons had already differentiated and become round/oval in shape and were approximately two times bigger than those in the ventricular zone. The lateral parts in the basal plate were devoid of neurons. Vacuoles were still present between the cells (Fig. 1.C)

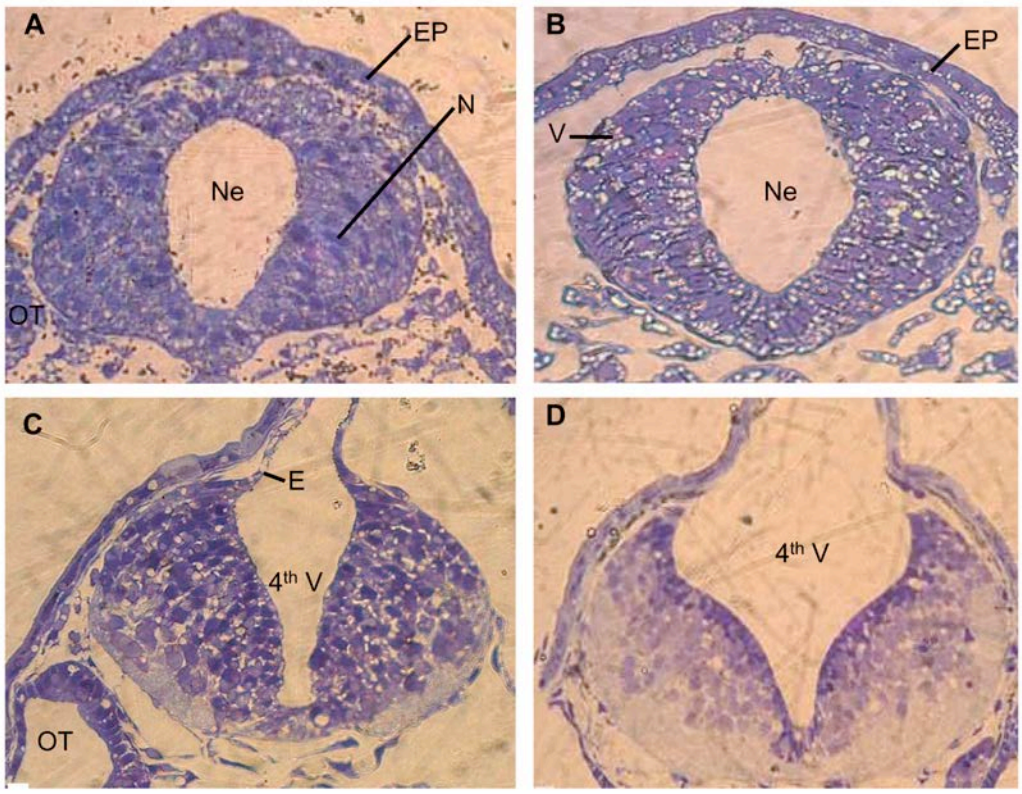

Figure 1: Developmental stages of the myelencephalon of the *Xenopus muelleri* tadpoles. (A-D) Show developmental stages (22, 25, 33, and 36) X 400, semi-thin sections stained with toluidine blue O. (A) Shows stage 22, in which neurons (N) are distributed in the entire myelencephalon surrounding the neural embryonic cavity, the Neurocoel (N). The Myelencephalon is located at the level of otic vesicle (OT) and is covered by an epidermis layer (EP). (B) shows developmental stage 25, with (V) representing the vacuoles. (C) show developmental stage 33, where the Neurocoel has developed to the 4th ventricle, which is lined by a single-celled layer of Ependymal (E). (D) Shows stage 36, where three quarter of the whole myelencephalon is occupied by the gray matter with the outer white matter devoid of neurons.

Stage 36: The gross shape of the myelencephalon anlagen was quite similar to that of the previous stage but the notable difference was that the neurons assumed a more elaborate oval shape and their size had increased to 12 µm. Three quarters of the whole myelencephalon was occupied by the gray matter with the outer white matter remaining empty without cells. Vacuoles were still present between the cells (Fig. 1.D).

Stage 40: At this stage the ependymal layer covering the roof of fourth ventricle was still intact but flat. Vacuoles have disappeared and most neurons were round in shape with the average diameter of (12.7 µm). Apart from the increase in size, there was a remarkable increase in number of cells. As noted in the previous stages, cells in the ependymal area were stained darker and smaller than those cells in the peripheral area, which tend to be larger and paler. The increase in number and size of cells was gradual and apparently due to mitosis and growth from a proliferation zone at the ventricular surface. Neurons were observed aggregating in groups limiting their spread to the core of the gray matter. The peripheral surface area remained without cells, thereby constituting the neuropil. The *sulcus medianus inferior* (smi) could also be distinguished as an indentation on the mid-ventral margin of the 4th ventricle. Below it there was well observed mass of round cells, which presumably constitute neurons of nucleus raphes (RP) of the median reticular formation (Fig. 2A).

Stage 44: At this stage the rhombencephalic roof is still intact but ependymal layer was stretched upward making the gross myelencephalon look like a lady's handbag. The arachnoid (A) surrounding the rhombencephalon could be traced as a thin black, delicate membrane (Fig. 2B). The structure of myelencephalon in general was more defined where the gray matter occupies two thirds of it while the remaining one third was occupied by white matter. Neurons were still in the differentiation and migration stages. However, no well defined cell masses were formed yet. Many of the large cells that were away from the ependymal area have assumed an oval shape with the average diameter of 13 µm. Mauthner cell (Mc), which is believed to be among the first neurons to differentiate, was observed at this stage. It is a large neuron with the average diameter of 30 µm (Fig. 2B). The *sulcus medianus inferior* was the only sulcus that was clearly distinguishable along the entire ventricular margin (Fig. 2A-C).

Stage 47 was notably different from the previous stages. The area of the myelencephalon that was occupied by cells was large due to the spread or migration of these cells from the ependymal area in the ventricular zone to their destination areas. Few cells could be recognized at this stage; nucleus *dorsalis nervi octavi* (VIIId) was situated in the dorsal lateral of the alar plate. The size of these cells was 7.3 µm. Nucleus *dorsalis nervi octavi* was formed by loosely aggregated cells that migrate in a ventro-lateral arc from the marginal zone of the alar plate to the peripheral zone (see arrow Fig. 2C). Other cell masses were nucleus *motorius nervi facialis* (VIIm) and nucleus *reticularis medius* (Rm). Mauthner cells had increased in size from their previous status by attaining a diameter of 36.6 µm. Another notable difference was the presence of the *sulcus limitans of His* (slH), which separates the dorsal alar plate from the ventral basal plate of the myelencephalon. The primordium of the choroid plexus (Cp) also expands and forms invagination into the fourth ventricle. The dorsal island (di) was clearly differentiated from the rest of the myelencephalon as a clear area on the latter part of the alar plate. The dorsal island represents a neuropil, i.e. an area which is occupied by a framework of interlacing dendrites and axonal terminal ramifications. Fig. 2D shows the level at which nerve number eight (nVIII) entering the brain and few cells of nucleus dorsalis nervi octavi (VIIId) were also observed.

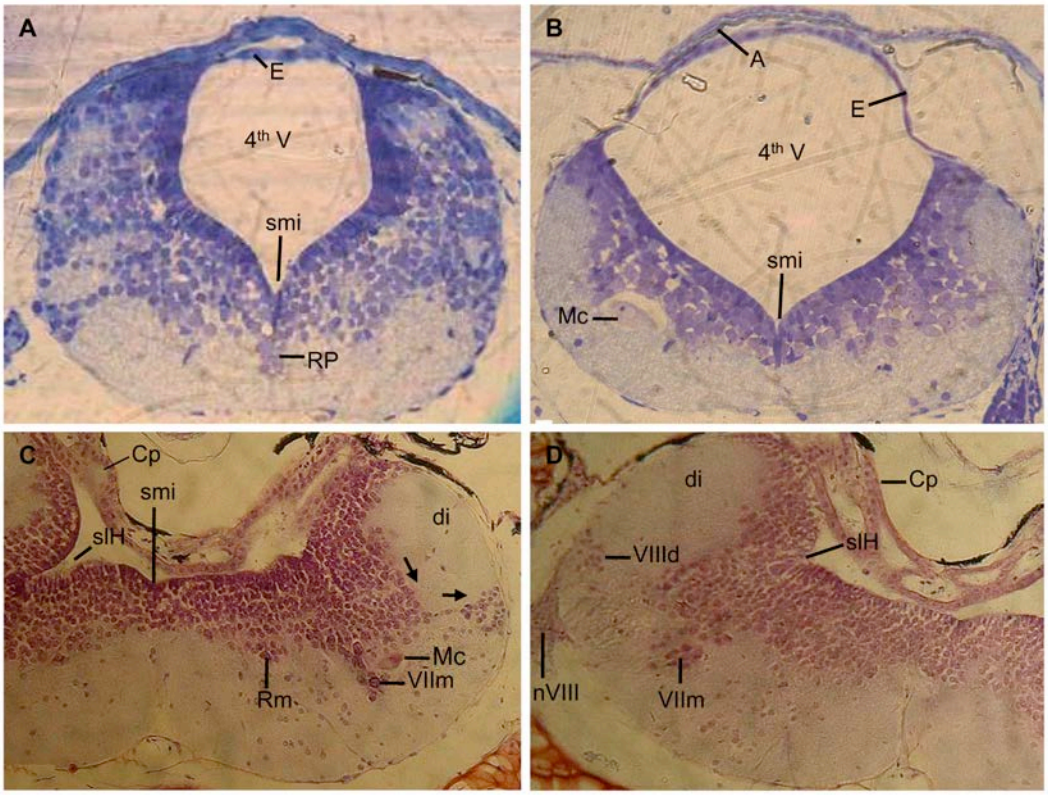

Figure 2: Developmental stages of the myelencephalon of the *Xenopus muelleri* tadpoles. (A-B) Show developmental stages (40 and 44) X 400, semi-thin sections stained with toluidine blue O. (A) Shows stage 40 with a small groove at the mid-ventral of the 4th ventricle (4th V), called *sulcus medianus inferior* (smi). Below the smi is a group of rounded neurons of the *Raphe* nucleus (RP). Ependymal layer (E) is seen as a flat membrane covering the 4th ventricle. (B) Shows stage 44. At this stage a Mauthner cell (Mc) is shown. Dorsal to the ependymal layer is a delicate membrane, the Archanoid (A). (C-D) Show Nissl stained sections of stage 47. Di represents the dorsal island. The black arrows show the migratory route of the nucleus *dorsalis nervi octavi* (VIIId). Lateral to the VIIId is nerve number eight (nVIII). Nucleus *motorius nervi facialis* (VIIm) and nucleus *reticularis medius* (Rm) are other structures, which could be identified. Lateral to the smi is *sulcus limitans of His* (slH) whereas the choroid plexus (Cp) is located on its dorsal side.

Stage 49/50: There was a continuous layer of cells from the ventricular zone to the mantle and marginal zones in the previous stages of development (Fig. 1 & 2). At stages 49/50 there was a notable difference where the myelencephalon was segregating into layers (Fig. 3A-B). A conspicuous layer of columnar ependymal cells occupies the first inner layer. This was followed by a narrow strip (*stratum subependymal)* which was practically devoid of cells and a continuous zone of central gray; stratum griseum where majority of the *neuronal perikarya* are located. The peripheral half wall of the rhombencephalon is supposed to be largely occupied by myelinated fibers without cells. However, at these stages, few cells of the olivary superior nucleus (Ols) and dorsal nucleus of nerve number eight (VIIId) were still scattered in this layer (Fig.

3A). In the alar plate, while the VIIId and nucleus *caudalis nervi lineae lateralis* (CII) were distinct nuclear masses, that can be hardly said of ventral nucleus of eight (VIIIv). This observation implies that at these stages, the myelencephalon was approaching the adult form. The migration and differentiation processes, however, were not yet over since some cells had not yet settled at their respective nuclei. Sensory fibers of nerve eight were seen entering the brain as in the previous stage (Fig. 3A).

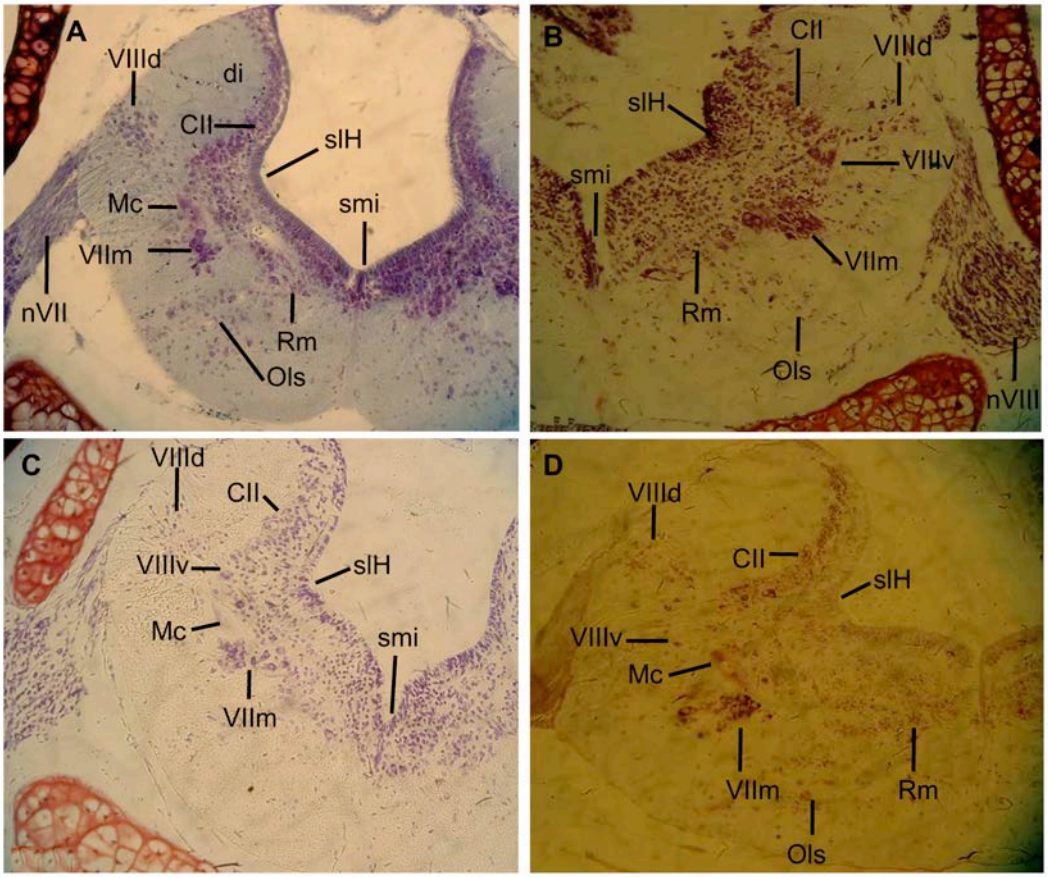

Figure 3: Developmental stages of the myelencephalon of the *Xenopus muelleri* tadpoles. (A-D) show Nissl stained sections of stages (49, 52, 54 and 59) X 200. Nomenclature of cell masses (nuclei), grooves and nerves are given. (A) Shows stage 49, whereas stage 52 is shown in (B), and stages 54 and 59 are shown in (C) and (D), respectively. The nuclei observed at these stages were; the nucleus *dorsalis nervi octavi* (VIIId), the ventral nucleus of eight (VIIIv), the nucleus *motorius nervi facialis* (VIIm), the nucleus *reticularis medius* (Rm), the olivary superior nucleus (Ols) and the nucleus *caudalis nervi lineae* lateralis (CII). Other structures include; the nerve seven (nVII), the nerve eight (nVIII), the sulcus limitans of His (slH), the sulcus medianus inferior (smi) and the Mauthner cell (Mc).

In Fig. 3B, a similar pattern as of stage 49 was seen in the myelencephalon of stage 52. Ventral nucleus of eight (VIIIv) somehow could be delineated from the

undifferentiated central gray. It seems that neurons that form the Ols broke away from the neuronal population that forms *nucleus reticularis medius*. In addition, many cells can be seen migrating to aggregate into respective nuclei.

Stage 54-59: From stage 54 to 59 (Fig. 3C-D), there were no significant changes compared to stage 52 (Fig. 3B). What could be observed was the fact that the cellular organization of the myelencephalon was more or less similar to that observed in the adult frog. The empty *stratum subependymale* was clearly defined, leaving the inner ependymal layer with columnar ependymal cells. The nuclei were more organized. This observation implies that from stage 54 (Fig. 3C) the cell masses in the myelencephalon were completely formed. What remained was the later development, consisting mainly of maturation and some further cytological differentiation.

DISCUSSION

General Features of Myelencephalon Development

The earliest tadpoles that were studied correspond to developmental stages 22 to 47 (Nieuwekoop and Faber 1956). At these late embryonic stages, no specific cell masses were distinguishable. Cell masses were formed in the latter half of larval stages (stages 48 to 59). Through metamorphosis especially from stage 59 onwards, the size and shape of neurons resembled those seen in adult animals (Fig. 3D). During the later stages the neurons seemed to settle on their respective nuclei. Hence, change of shape and size of neurons was a gradual development process and did not coincide with large morphological and physiological changes that took place at metamorphosis. Vacuoles that were observed in developing myelencephalon disappeared at stage 40. Van Mier (1986) observed the disappearance of yolk granules and vacuoles during larval development of *Xenopus laevis*. In all tadpoles, the rhombencephalic roof was intact. It was covered by a thin delicate single-celled ependymal layer. Brocklehurts (1976) found the same region intact in tadpoles of *Rana temporaria.*

Neuronal Migration and Formation of Cell Masses

The density of neurons increased as the larva developed. This suggests that dividing nerve cell precursors continuously add new neurons to the pre-existing ones, during larval stages. Recognition of presumptive nuclear mass started at stage 40 during which time neurons in the ventral part of the myelencephalon begin to segregate to form the presumptive raphes nucleus and nuclei of the reticular formation. These observations were supported by similar findings Van Mier, (1986) in *Xenopus laevis*. One of the earliest neurons that can be distinctly recognized from the mantle are the two-Mauthner cell, each of which was located in the ventro-lateral half of the rhombencephalon. In the dorsal part of this area of the brain the first to be identified were neurons that will later form the dorsal nucleus of VIIIth. In general, neurons in the mantle area were larger compared to those of the ependymal area. It was difficult, however to recognize in a given larval cell population the forerunner of an adult structure. In spite of these problems, it was possible to identify several components of the myelencephalon anlagen with the adult counterparts on the basis of a similar position.

The myelencephalon anlagen of the newly hatched tadpole stages 22-33 consists of post mitotic neurons, which were oblong in shape. The neurons were distributed throughout its mass, attached to dense meshwork processes, which are radially oriented. These cells presumably are migrating in these processes. But it was

difficult to establish exactly if these processes were glia fibers which, extend from ventricular zone to the pial surface. Also it was difficult to tell exactly which, mode of migration these neurons played whether it was radial glia or nuclear translocation into preformed pial processes. The "inside out" pattern of origin, which normally is observed in other structures of the Central Nervous System (CNS), was not observed in the myelencephalon. This suggests that this mode of migration was probably specific for layered structures of CNS (cerebellum and cerebral cortex).

In order to demonstrate a freely migrating cell in the developing nervous system, one would have to label the cell and follow it sequentially with reference to some fixed point, which was out of scope of this study. These results suggests that in earlier stages of the development of the myelencephalon of *Xenopus muelleri*, the neurons migrate from the ependymal layer in the ventricular zone either along the radial glia fibers or by perikaryal translocation to the mantle/marginal zones in the peripheral areas. Various hypotheses have been proposed to describe the migration of immature neurons. The most widely accepted is the radial glia hypothesis (Rakic 1971, 1972 and 1990). According to this theory, newly post mitotic neurons acquire a bipolar shape and lose their attachments to both the ventricular and pial surfaces. Instead they contact processes of radial and migrate along them to their final destinations. Thus, radial glia serves both as guides and as substrates for neuronal migration.

In the later stages of development, the meshwork type of fibers was no longer visible but the neurons were observed to continue with the process of migration to their final destinations in their respective nuclei. This implies that these neurons were no longer depending on fibers but rather on something else. Probably they depend on the interactions between neurons of the same group, which enables them to reach their final destinations and hence to form the nuclei. Based on histological techniques, large neurons migrate first before small neurons and motor nuclei migrate before sensory ones during the development. By stage 54, the structure of myelencephalon resembled that of the adult frog. What remained in later development constituted mainly of maturation and some further cytological differentiations. In order to yield more information about the origin, migration and the destination of nuclei, combination of normal histology and silver staining techniques will be the best option. The growth and differentiation of individual neurons in such nuclei, in successive stages of development can be studied over time to record when and how the adult form is attained.

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