Development of Granule Cells of the Rat Olfactory Bulb: An Autoradiographic and Electron Microscopic Study

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Summary. The rate of migration of immature granule cells of the rat olfactory bulb and polarity of cell organelles in the migrating granule cells were investigated by 3H-thymidine autoradiographic and electron microscopic methods. The time lag in migration between two points was determined by cross-correlation analysis of labeling indices of the two areas. Granule cells were estimated to take 6 days to migrate rostrally from the subependymal layer at the anterior wall of the lateral ventricle to the center of the bulb, and an additional 1 to 6 days to migrate radially from the subependymal layer to the granular layer of the bulb. These results showed that the rate of rostralward migration of granule cells was faster than that of their radial migration. Golgi-electron microscopic as well as routine electron microscopic studies on migrating granule cells revealed that centrioles and Golgi apparatus were located at the base of the leading process that possesses a growth cone at its tip.

The subependymal layer around the anterior horn of the lateral ventricle generates the granule cell of the olfactory bulb in the rodent (Altman, 1969; DeBassio and Kemper, 1985; Kishi, 1987; Frazier-Ciepial and Brunjes, 1989), in the carnivore (Wahle et al., 1990) and in the primate (McDermott and Lantos, 1990). Our previous reports have described two phases of migration of immature granule cells in the rat olfactory bulb (Kishi, 1987; Kishi et al., 1990). The first phase is the rostralward migration of bipolar granule cells in the subependymal layer; these originate in the subependymal layer around the anterior horn of the lateral ventricle, migrate through the subependymal layer along the olfactory ventricle, and reach the center of the bulb. The second phase is the radial migration from the subependymal layer to the granular layer of the olfactory bulb where immature granule cells differentiate into definitive granule cells. Bayer (1983) has established the generation period of the rat granule cells: 41% of them originate during the first postnatal week, 23% during the second week, and 7% during the third week. The rate of migration of the immature granule cells in the first phase has been studied in rats 35-41 days in age (DeBassio and Kemper, 1985). However, the rate in the first phase during the peak period of their generation, and the differences in rates between the first and the second phase during the same developmental stage, remain to be elucidated. Ultrastructural features of migrating granule cells, especially the polarity of the cell organelles, have not yet been studied.

This report describes the rate of migration of immature granule cells during the peak period of their generation. The polarity of cell organelles in these cells is also studied by electron microscopy.

MATERIAL AND METHODS

Wistar rats 6, 7, 9, 12 and 14 days of age were used in this study, day 1 representing the day of birth. The animals were kept in cages in a room with 16/8 light/dark cycle and given laboratory chow and water ad libitum.

Autoradiography

Rats of 6 days of age were given an intraperitoneal injection of 3H-thymidine (4 μCi/g body weight; New England Nuclear, specific activity 10-30 Ci/mM). At 1, 3, 6 and 8 days after the injection, the animals were anesthetized with an intraperitoneal injection of 3.5% chloral hydrate (1 ml/100 g) and perfused through the heart with 10% formalin in 0.1 M sodium...
phosphate buffer (pH 7.4). Brains were removed and fixed with the same fixative for 1 day. They were then dehydrated and embedded in paraffin. Serial coronal sections were cut at 8 μm, and mounted on slides. They were then dipped in NTB-2 emulsion (Kodak), placed in light-tight boxes with a desiccant, exposed at 4°C for 20-35 days, developed in D-19 (Kodak), and counterstained with 0.01% thionin. The number of labeled cells per unit area of the subependymal layer was counted at 6 levels ranging from the anterior horn of the lateral ventricle to the anterior part of the olfactory bulb at each stage of development for the rats. The number of labeled cells in the granular layer was also counted at the middle level of the olfactory bulb. Quantifications for all groups were made with a 100X oil immersion objective and a 10X ocular containing a grid reticule. A cell was considered “labeled” if four or more grains were found over its nucleus. The density of all cells in the subependymal and granular layer was also counted at each level studied. From these data, labeling indices, i.e., the number of labeled cells per 100 cells, at each level, were calculated at each survival time after injection.

Analysis of the time lag of migration

The cross-correlation analysis is commonly used for statistical estimation of association between two data of time-series. We have employed this method to analyze the time lag of migration between 2 levels (SHIMAZAKI, 1989). The time-series of labeling indices at a certain level (Lx) was defined as X consisting of x1, x2, x3, ..., xn values at survival times of 1, 2, 3, ..., n. The time-series of labeling indices at a different level (Ly) was also defined as Y consisting of y1, y2, y3, ..., yn. A cross-correlation analysis allows to measure the magnitude of the influence of X on Y. Cross-correlation coefficient of \( \{x_i, y_i\} \) (i=1, 2, ..., n) was defined as \( r_{xy} \), \( \{x_i, y_{i+1}\} \) (i=1, 2, ..., n-1) as \( r_1 \), and \( \{x_i, y_{i+k}\} \) (i=1, 2, ..., n-k) as \( r_k \). If X significantly influences Y after a time lag of k, the value of \( |r_k| \) increases. Time patterns of the influence of X on Y can be estimated from a correlogram of \( |r_k| \) plotted as a function of time lag of k. The coefficient of \( r_k \) was calculated from the following formula:

\[
\begin{align*}
\tau &= \frac{\sum_{i=1}^{n-k} x_i y_{i+k} - \sum_{i=1}^{n-k} x_i \sum_{i=1}^{n-k} y_{i+k}}{\sqrt{\left( \sum_{i=1}^{n-k} x_i^2 - \sum_{i=1}^{n-k} (\sum_{i=1}^{n-k} x_i)^2 / (n-k) \right) \left( \sum_{i=1}^{n-k} y_i^2 - \sum_{i=1}^{n-k} (\sum_{i=1}^{n-k} y_i)^2 / (n-k) \right)}}
\end{align*}
\]

Significant differences among cross-correlation coefficients were examined by Student’s t-test (n=2 to 5).

For measurement of length of migratory stream, 5 rats of 6, 7, 9, 12, and 14 days in age were perfused with 10% buffered formalin, and cut in serial frozen sections at a thickness of 30 μm along the coronal planes. The speed of migration of young granule cells was estimated from the data of time lag of migration between two levels and the distance of migration.

Electron microscopy

For electron microscopic observation of Golgi-impregnated cells, 6-day-old rats were perfused through the heart with a solution of 4% paraformaldehyde, 0.5% glutaraldehyde, 0.54% dextrose in 0.1 M sodium phosphate buffer (pH 7.4) (CRAIN et al., 1973). Brains were fixed with a potassium dichromate mixture (STENSAAS and STENSAAS, 1968) for 2 days at 4°C, and immersed in 0.75% silver nitrate solution for 2 days at 4°C. The subependymal layer was cut sagittally at a 100 μm thickness using a Vibratome. The sections containing suitably impregnated subependymal cells were selected under the light microscope, and dipped in glycerol. Such sections were successively immersed in solutions of gold chloride, of oxalic acid, and of sodium thiosulfate. They were then postfixed with 1% OsO4 for 30 min at room temperature, and embedded in Epon. Ultrathin sections were observed under the electron microscope (FAIREN et al., 1977). In addition to the electron microscopic study of Golgi impregnated subependymal cells, the 6-day-old subependymal layer fixed with the fixative of CRAIN et al. (1973) and embedded in Epon were also observed with routine transmission electron microscope.

RESULTS

3H-thymidine autoradiography

Nissl stained sagittal sections of 6 to 14-day-old rats have shown that the subependymal layer composed of a darkly stained cell mass around the ependymal layer of the anterior lateral ventricle extends rostrally along the course of the olfactory ventricle to the center of the olfactory bulb where the subependymal layer is surrounded by the granular layer (Fig. 1).

Table 1 summarizes mean values of labeling indices at each survival time after 3H-thymidine injection. Labeling indices in the frontal cortex, in the caudate-putamen, and in the anterior olfactory nucleus in the olfactory peduncle are around 1% at 1 h after injection, and remained at 2-5% throughout the period of this study in comparison with the high values of indices in the subependymal layer and in the granular layer of the bulb.
Fig. 1. Photomicrograph of sagittal section of a Nissl stained 9-day-old rat brain showing a darkly stained subependymal layer (arrow) and levels (L₁-L₆) of autoradiographic analysis. GRL: granular layer of the olfactory bulb. Bar=500μm

Table 1. Labeling indices in ³H-thymidine autoradiogram at each level

<table>
<thead>
<tr>
<th></th>
<th>Survival after injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>SEL at L₁</td>
<td>15.5 ± 0.7</td>
</tr>
<tr>
<td>SEL at L₂</td>
<td>9.0 ± 1.4</td>
</tr>
<tr>
<td>SEL at L₃</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>SEL at L₄</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>SEL at L₅</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>SEL at L₆</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>GRL at L₁</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>FCX</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>C-P</td>
<td>1.2 ± 1.2</td>
</tr>
<tr>
<td>AON</td>
<td>0.8 ± 0.2</td>
</tr>
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Values represent mean percentages of labeled cells±SD.
Labeling indices of the subependymal layer were counted at the following 6 levels of coronal sections (Fig. 1): level of the anterior horn of the lateral ventricle where the triangular subependymal layer was bordered by three structures, i.e., the corpus callosum, the caudate-putamen, and the septum (L1); level of the rostral wall of the lateral ventricle where the subependymal layer was bounded by the forceps minor of the corpus callosum, by the caudate-putamen, and by the medial wall of the cerebral hemisphere showing tenia tecta (L2); the middle level of the olfactory peduncle (L3); the caudal level of the olfactory bulb (L4); the middle level of the olfactory bulb (L5); and the rostral end of the subependymal layer in the olfactory bulb (L6). Figures 2 and 3 show distributions of labeled cells in the subependymal layer at L2- and L5-levels. The density of labeled cells in the subependymal layer at L2-level is highest at day 1. Peaks of labeling indices at L1, L3, L5 and L6 appear at 1 day, 3 day, 6 days, and 6 days, respectively, after injection. Labeling indices are high at levels of L4-L5 at 8 days after injection (Table 1). The time lag of subependymal cell-migration between L2 and L3 was analyzed by cross-correlation analysis (Fig. 4). The coefficient (0.984) of the cross-correlation at the time lag of 1 day is significant (p<0.01), meaning that it takes 1 day to migrate from the rostral wall of the lateral ventricle (L2) to the middle level of the olfactory peduncle (L3). The coefficient for migration from L2 to L5 shown in Figure 5 to be significant at the time lag of 6 days (0.999, p<0.05). The coefficient for migration from L2 to L6 is also significant at the time lag of 6 days (0.999, p<0.05, figure not shown). These results indicate that it takes 6 days to migrate in the subependymal layer from the rostral wall of the lateral ventricle to the middle and the rostral parts of the olfactory bulb. The length of the subependymal layer is 3300 µm between L2 and L5, and 4700 µm between L2 and L6 in 12-day-old rats. The rate of migration is estimated to be 23 µm/h between L2 and L5, and 33 µm/h between L2 and L6.

Figure 6 shows the time-course of labeling indices of subependymal and granular layers at the middle level (L5) of the olfactory bulb. Labeling indices in the subependymal layer remain low during the first day after injection, increase to 19±14.1 at 3 days, reach the peak (46.5±2.1) at 6 days, and decline to 33.1±10.8 at 8 days. Labeling indices in the granular layer...
Fig. 4. Correlogram of labeled subependymal cells at L2- and L3-levels. Abcissa: time-lag, ordinate: cross-correlation coefficient, **p<0.01

Fig. 5. Correlogram of labeled subependymal cells at L2- and L5-levels. Abcissa: time-lag, ordinate: cross-correlation coefficient, *p<0.05

Fig. 6. Percentage of labeled cells in the subependymal layer (solid line) and in the granular layer (broken line) at L5 level of the olfactory bulb. Abcissa: survival after injection, ordinate: percentage of labeled cells.

Fig. 7. Correlogram of labeled subependymal cells and of granule cells at L5-levels. Abcissa: time-lag, ordinate: cross-correlation coefficient, *p<0.05
Fig. 8 A. Photomicrograph of Golgi-stained subependymal layer of 6-day-old rat showing bipolar subependymal cells, i.e., young granule cells of the olfactory bulb. B. Photomicrograph of bipolar subependymal cells after de-impregnation and gold toning for Golgi-electron microscopy. Note the long leading process and short trailing process. C. Electron micrograph of Golgi-stained bipolar subependymal cell marked by electron-dense particles of gold. Note the Golgi-apparatus (g) at the base of the leading process. D. Electron micrograph of a growth cone (surrounded by arrowheads) of a bipolar subependymal cell. Bars = 20 μm for A, B; 1 μm for C, D.
Fig. 9. Electron micrographs of a sagittal section of bipolar subependymal cells, i.e., young granule cells of the olfactory bulb. A. Cell body showing trailing process (t) and a leading process with Golgi apparatus (g). B. Base of leading process showing centriole (c) and Golgi apparatus (g). C. Growth cones (arrows) containing many vesicles and fuzzy substance. Bars=1 μm.
also remain very low during the first 3 days after injection. These increase to 20.5±3.5 at 6 days, and reach the peak (34.3±5.3) at 8 days of survival. Time lag for migration from the subependymal to the granular layers of the olfactory bulb was also analyzed by cross-correlation analysis (Fig. 7). Coefficients are significant at the time lag of 1 day (0.974, p<0.05) and of 6 days (0.999, p<0.05). The coefficient at the time lag of 3 days tends to be high (0.877). The results indicate that it takes at least 1 day to migrate from the subependymal to the granular layers of the olfactory bulb. The migration between two layers continues for 6 successive days. The radius estimated from major and minor axes of the subependymal layer at L5 level is 400 μm in 12-day-old rats, and becomes shorter with age (300 μm in 14-day-old rats). According to our former Golgi study (KISHI, 1987), bipolar granule cells show a marked trend to shift the orientation of their leading processes towards the granular layer from the mid-level of the subependymal layer, i.e., 200 μm away from the border of the granular layer in 12-day-old rats. Assuming that it takes 1-6 days to migrate the distance of 200 μm, the rate of radial migration of granule cells is 8.3-1.4 μm/h. Labeled cells in the granular layer tend to distribute in the deeper half of the layer at 3 and 6 days after injection (Fig. 3). The width of the granular layer is 500 μm in 12-day-old rats. Even if granule cells migrate from the mid-level of the subependymal layer to the mid-level of the granular layer for 1 day, the rate of migration is estimated to be 18.8 μm/h.

**Electron microscopy**

A sagittal, Golgi-stained section of the subependymal layer around the olfactory ventricle showed many bipolar cells with a long leading process tipped by a growth cone and with a short trailing process (Fig. 8A). To elucidate the ultrastructural features of migrating bipolar cells, we observed them with Golgi-electron microscopy. Bipolar cells became semitransparent after deimpregnation and a gold-toning procedure (Fig. 8B). These cells could be identified by distribution of fine gold particles under the electron microscope. They appeared dark in the cytoplasm and Golgi apparatus at the base of the leading processes. Their growth cones showed thin filopodia extending in the intercellular space between other subependymal cells (Fig. 8C, D). Under the usual transmission electron microscope, bipolar cells could be identified by a dark appearance with many free ribosomes. Sagittal sections of these cells also revealed a Golgi apparatus as well as centrioles at the base of the leading processes in contrast to the absence of a Golgi apparatus in the vicinity of the trailing processes (Fig. 9A, B). Microtubules were abundant in the leading processes and scanty in the trailing processes. Free ribosomes, smooth endoplasmic reticulum, and vesicles were found among microtubules in the leading processes. Growth cones showed many vesicles at their proximal parts and a fuzzy substance in the filopodia (Fig. 9C).

**DISCUSSION**

The present autoradiographic study using rats injected with ³H-thymidine on postnatal 6 days has clearly shown that labeling indices in the neighboring regions of the rostral subependymal layer, i.e., the frontal cortex, the caudate-putamen, and anterior olfactory nucleus, remain constantly low in contrast to the high indices in the subependymal layer immediately after injection, and those in the granular layer of the olfactory bulb on subsequent days. Neurogenesis has been reported to cease in the rat cortex at 21 embryonic days (BAYER and ALTMAN, 1991), in the anterior olfactory nucleus on postnatal day 1 (BAYER, 1986), and in the caudate-putamen on postnatal day 2 (FENTRESS et al., 1981). These results suggest that the majority of labeled cells in the subependymal layer around the anterior horn of the lateral ventricle on the 6th day are immature granule cells (ALTMAN, 1969; KISHI, 1987), and that a small number of labeled cells migrate towards the neighboring regions, except the olfactory bulb.

Our previous reports (KISHI, 1987; KISHI et al., 1990) have described two phases of migration of immature granule cells in the olfactory bulb. The first phase is the rostralwards migration of postmitotic precursor granule cells in the subependymal layer. The second phase is the radial migration from the subependymal layer to the granular layer in the olfactory bulb where immature granule cells differentiate into definitive granule cells.

The present electron microscopic study on migrating immature granule cells in the first phase has elucidated the following facts: the Golgi apparatus and centriole are located at the base of leading process; the leading process contains a massive bundle of microtubules; vesicles and smooth endoplasmic reticulum are found among microtubules of the leading process and at the base of the growth cone. These results are apparently consistent with the following reports on migrating cells. In interphase cell, the Golgi apparatus is usually found in proximity to the microtubules, organizing a center and comprising a pair of centrioles surrounded by a pericentriolar material which has the ability to nucleate microtubules (CAJAL, 1960; WENT-
The Golgi apparatus and microtubule organizing center are rapidly repositioned forewards of the nucleus in the direction of migration at the moment of cell motility (KUPFER et al., 1982). The Golgi apparatus may be involved in the processes of inserting a new plasma membrane to the growth cone, and that of recycling of membrane. The microtubule organizing center and microtubules are considered involved in providing a tracking system for directing the traffic of the Golgi-apparatus-derived vesicles to the growth cone (BERGMAN et al., 1983).

The present cross-correlation analysis on the time lag of migration in the first phase has shown that it takes 6 days to migrate from the rostral wall of the lateral ventricle to the center of the olfactory bulb during the peak period of granule cell generation (BAYER, 1983). It has been estimated that the rate of migration of immature granule cells in the first phase is 23-33 \( \mu \text{m/h} \) in rats 6-12 days in age. This rate is roughly comparable to the following findings: namely rates of migration of granule cells of the olfactory bulb in the first phase 100 \( \mu \text{m/h} \) in 25%-casein-diet rats of 35-41 days in age, and 33-70 \( \mu \text{m/h} \) in 8%-casein-diet rats of the same age (DEBASSIO and KEMPER, 1985). By contrast, slower rates of migration are reported for cerebellar granule cells of the mouse (4.2 \( \mu \text{m/h} \), FUJITA et al., 1966), for neurons of the cortical plate (5.5 \( \mu \text{m/h} \), RAKIC, 1974), for neurons of chick optic tectum (4 \( \mu \text{m/h} \), LAVAIL and COWAN, 1971), and for neurons of the chick isthmo-optic nucleus (4 \( \mu \text{m/h} \), CLARKE and COWAN, 1976). These results indicate that the rostralward migration of immature granule cells is far more rapid than for other neurons. Definitive explanations for these striking differences in the rate of migration of immature granule cells are not readily apparent, although one possible explanation is that immature granule cells have a leading process with an unusually large growth cone acting as a great motile force for migration (KISHI, 1987). Another explanation may be the abundance of extracellular spaces throughout their course of migration in the subependymal layer, which is similar to the condition of cultured cerebellar granule cells that show a migration-rate of 33\( \pm \)20 \( \mu \text{m/h} \) (KISHI et al., 1990; EDMONDSON and HATTEN, 1987).

Concerning the second phase of migration of granule cells, it has been elucidated that the time required for this radial migration from the subependymal layer to the granular layer of the olfactory bulb is 1 to 6 days. Thus, it takes 7 to 12 days to migrate from the subependymal layer around the rostral wall of the lateral ventricle to the granular layer of the olfactory bulb. The rate of migration is estimated to be 18.8 to 1.4 \( \mu \text{m/h} \). The minimum speed of this migration is roughly consistent with the values given by ALTMAN (1966) reporting that the rate of migration from the olfactory ventricular wall to the mitral cell-layer of rat is 2.1 \( \mu \text{m/h} \). The rate of migration in the second phase is apparently slower than that of the first phase. This may be explained from our former observation of Golgi-impregnated young granule cells (KISHI, 1987). Leading processes of young granule cells begin to vary direction at the mid-level of the subependymal layer of the bulb, and gradually orient obliquely or vertically to the border of the granular layer. These findings suggest that young granule cells initially search for the direction of their migration at the mid-level of the subependymal layer, and that this process of searching for the direction might cause stagnation of their migration. Growth cones at the tip of the leading processes of young granule cells become smaller after invasion into the granular layer. In addition, extracellular space in the granular layer was very limited during the developmental stages of this study (unpublished observation). The present autoradiographic study has clearly shown that younger neurons previous to their maturation migrate more rapidly than the neurons moving into the vicinity of the target place where they differentiate into a definitive form.

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