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IS CEREBELLAR GRANULE CELL MIGRATION REGULATED BY AN INTERNAL CLOCK?[1]

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Abstract

We have studied the time course of migratory behavior of cerebellar granule cells in the microwell tissue culture system. [^{3}H]Thymidine served as a marker for particular granule cell generations. When cultured 4 hr after [^{3}H]thymidine injection for 6 days in microwell cultures, labeled granule cells were seen to migrate along fiber bundles expanding between reaggregates called "cables" for 3 to 4 days. After 5 and 6 days *in vitro* the percentage of labeled non-migrating cells found in clusters in reaggregates and on cables increased considerably. Whereas unlabeled cells continued to migrate. Comparable results were obtained when granule cells developed *in vivo* for various times after label and their developmental state was determined *in vitro*. Cells from cerebellar populations labeled 1 to 4 days before culture maintained their ability to migrate *in vitro*, even after granule cells had entered the internal granule cell layer. In contrast, the percentage of migrating cells labeled 5 and 6 days before culture was reduced significantly. The results suggest that the time span of granule cell migration is predetermined intrinsically rather than by external signals.

Cells in the developing central nervous system (CNS) are formed in specialized germinal zones and obtain their final position through active migration. The migratory process is thought to be governed in part by genetically and epigenetically defined sequences of signals which are interpreted by migrating cells. This study attempts to determine the developmentally important signals for the timing of cerebellar granule cell maturation as defined by cell migratory behavior and interactions with other cells *in vitro*.

During the initial period of proliferation, all external granule cells (EGCs) appear to be engaged in DNA synthesis and mitosis. In the cerebellum pulse labeling with [^{3}H]thymidine has been used to determine the birth dates of cerebellar neurons and to describe the period of cell proliferation of particular granule cell generations (Miale and Sidman, 1961; Fujita, 1964, 1967; Fujita et al., 1966; Altman et al., 1982). In the past decade, Rakic and others (Rakic, 1971, 1974, 1978, 1981; Caviness and Rakic, 1978; Levitt and Rakic, 1980) have established that, in the CNS, immature migrating neurons are closely associated with radial glia cell processes. Time of origin and route of migration of immature granule cell neurons have been described in a large number of studies (Ramón y Cajal, 1929; Miale and Sidman, 1961; Fujita, 1964, 1967; Fujita et al., 1966; Altman, 1982). After their last mitosis granule cells move into a post-mitotic zone where they wait for 21 to 24 hr and then migrate into deeper layers of the cerebellar cortex. The migration of one generation of EGCs from the external granule cell layer (EGL) into the internal granula cell layer (IGL) takes about 35 to 40 hr in the mouse (Fujita et al., 1966; Fujita, 1967).

We have designed a microwell tissue culture system of early postnatal mouse cerebellum to analyze cell movements and cell interactions (Trenkner and Sidman, 1977). Within hours after being placed in culture cerebellar cells form reaggregates which later develop interconnections consisting of bundles of cell processes, here referred to as cables. Presumptive granule cells migrate along the cables (Trenkner and Sidman, 1977). This *in vitro* system allows for the analysis of the dynamics of cell behavior important to histogenesis.

The aim of this study is to analyze the timed sequence of granule cell maturation and to determine whether this sequence is intrinsic to granule cells or dependent upon environmental signals.

Materials and Methods

Microwell tissue culture. Single cell suspensions of early postnatal mouse cerebellum (C57BL/6J) were prepared as described earlier (Trenkner and Sidman, 1977). Cells (5×10^4) of 3-day-old mice were cultured in microtiter plates (Falcon 3034) for 3 and 6 days, respectively. Cells were maintained in Eagle's basal medium supplemented with 10% horse serum, glutamine, penicillin, and streptomycin as detailed elsewhere (Trenkner and Sidman, 1977).

Time lapse cinematography. Migratory behavior of granule cells was monitored *in vitro* over a period of 6 days by time lapse cinematography as described previously (Trenkner and Sidman, 1977).

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Thymidine label. Three- to 7-day-old (P3 to P7) C57BL/6J mice were immobilized on ice and injected subcutaneously with 5 μl of [^{3}H] thymidine ([^{3}H]Thy) (25 Ci/mmol, Amersham Searle). At given times after label, cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) and 0.25% glutaraldehyde, washed three times in PBS, and dehydrated in ethanol. To determine the number of labeled cells in single cell preparation, cells were centrifuged and the pellet was fixed, dehydrated, and embedded as described before. If necessary, cells were pelleted after each step by centrifugation (1000 rpm). Subsequently, cultures were subjected to autoradiography.

Autoradiography. Approximately 3 μl of a 1:3 dilution of Ilford G-5 emulsion were added per microwell, dried, and exposed for 4 weeks at −70°C. Cultures were counterstained with 1% toluidine blue, and the number of labeled versus unlabeled cells on cables was determined. To determine the distribution of labeled cells in reaggregates, cultures of [^{3}H]Thy-labeled cells were fixed, dehydrated and embedded in Epon as described previously (Trenkner and Sidman, 1977). One-micrometer sections were coated with a 1:2 dilution of Ilford G-5 and exposed for 6 weeks at −70°C. Sections were counterstained with toluidine blue.

Scanning electron microscopy. After fixation and dehydration in ethanol, cultures were critical point dried with liquid CO_2 and coated with carbon and gold palladium (Boyde et al., 1977).

Results

Behavior of migrating cells in vitro. In earlier work cell migration was studied by time lapse cinematography in a microwell tissue culture system (Trenkner and Sidman, 1977). Morphological characterization of migrating cells suggested that these cells are granule cell neurons (Trenkner and Sidman, 1977). In this study we attempted to relate cell shape to migrating behavior in order to distinguish migrating cells from non-migrating cells in fixed cultures.

The state of motion of 500 migrating cells was analyzed in time lapse movies over a period of 5 days and grouped into three categories: "running," "intermediate," and "standing." One typical example of a cable with all three types visible on it is illustrated in Figure 1. The presumptive granule cell neurons migrate along cables consisting of parallel fiber fascicles and glial processes (Liem et al., 1982). The speed of migration of individual cells varies considerably between 0.5 and 25 μm/hr. Occasionally they change directions, in certain cases up to four times, and rotate around the cable.

The shape of a cell is related to its state of motion. Running (r) cells (10 to 25 μm/hr) were stretched along the cable and had a drop-like shape, with the thinner pole pointing toward the direction of migration (Fig. 1). Intermediate (i) cells (0.5 to 5 μ/l) were rounder but remain attached to the cable over the length of their cell body (5 to 6 μm) (Fig. 1). Migrating cells pass each other and non-migrating cells, apparently uninfluenced by them. The number of migrating cells was approximately the same throughout the culture period of 6 days. This indicates that the decline of migratory activity of particular granule cell generations does not reflect a change in culture conditions.

There are also cells which are permanently postmigratory. We call these "standing" cells. They are round, barely attached to cables, and clustered together. This might suggest that the surface properties of granule cells differ with the migratory state (Fig. 1). Postmigratory (standing) cells were never seen to resume migration.

Migrating cerebellar cells in vitro are granule cells. The vast majority of cells (95%) dividing postnatally in the cerebellum are external granule cells (Miale and Sidman, 1961; Fujita, 1964, 1967; Fujita et al., 1966; Altman, 1982). Therefore, [^{3}H] Thy provides a useful marker to follow the development of particular granule cell generations. We define a generation of granule cells as those cells which enter the postmitotic state at the same time.

P3 mice were injected subcutaneously with [^{3}H]Thy once. Twenty-three to 28% of EGCs and 9 to 12% of cells in the IGL were labeled 4 hr after injection. No cells in the molecular layer (ML) were labeled at this time. Comparable numbers of labeled cells (23 to 26%) were observed in freshly prepared single cell suspensions, as determined in 1-μm sections of the cell pellet.

After 2 to 3 days in culture a considerable number of [^{3}H] Thy-labeled cells appeared on cables as demonstrated in Fig-

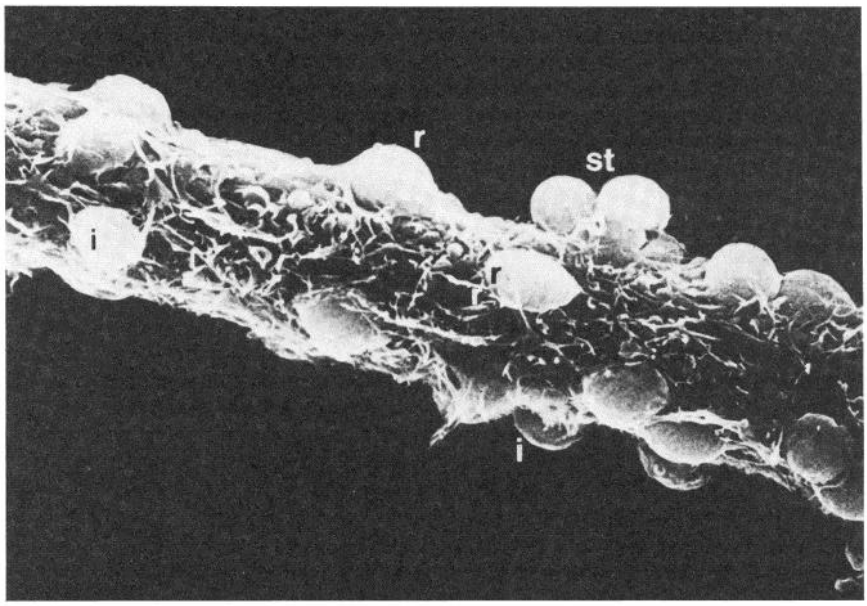

Figure 1. Scanning electron micrograph of migrating granule cells on a "cable." The shapes of the cells reflect their migrating activity. *r*, Running or fast moving cells (10 to 25 μm/hr) were stretched along the cable. *i*, Intermediate or slow moving cells (0.5 to 5 μm/hr) were rounded up but remained attached to the cable over the length of their cell body. *st*, Standing cells did not migrate and have never been observed to migrate again. They rounded up barely attached to the cable and formed contacts between each other. Magnification × 2000.

ures 2 and 3. Since *in vivo* nearly all labeled cells are granule cells, this, taken together with results from earlier studies (Trenkner and Sidman, 1977) suggests that migrating cells in the microwell tissue culture system are granule cells.

Timing of the sequence of granule cell maturation in vitro. To determine whether granule cells maintain their developmental schedule under tissue culture conditions the development of granule cells was analyzed *in vitro* and the timing of the sequence of developmental changes was compared with that of granule cells *in vivo*. EGCs were labeled at P3 and put into culture 4 hr later. Cultures were maintained for 7 days, and the number of labeled cells on cables and in reaggregates was determined by autoradiography at daily intervals (Figs. 3 and 5*B*).

In vivo, 35 to 40 hr after injection the labeled granule cells increase in number in the molecular layer (ML), and after 48 to 52 hr they reach the IGL (Fujita, 1967; E. Trenkner, unpublished observation). After 2 days *in vitro* approximately 26% of cells migrating on cables were labeled. This increased to 35 to 37% after 3 days *in vitro*. The greatest percentage of labeled cells is found on cables after 72 to 96 hr, since the fraction gradually decreases after 96 hr in culture. After 6 days, when more than 95 to 98% of all labeled granule cells *in vivo* are found in the IGL, only 8 to 14% of the labeled cells *in vitro* were found on cables. They were mostly in a standing state or aggregated on the cables. On the other hand, unlabeled cells continued to migrate, indicating that the conditions for migration were maintained throughout the period of culture.

The percentage of labeled cells in reaggregates was determined in 1-μm sections. Twenty-six to 30% of the granule cells were labeled after 2 days *in vitro*. Most of them were scattered throughout the reaggregate (Fig. 3*A*). With time in culture the percentage of labeled granule cells in reaggregates decreased to approximately 14%, at a time when the majority of labeled granule cells migrated on the cables. However, after 5 to 6 days in vitro, the percentage of labeled granule cells in reaggregates increased again to approximately 23 to 28%. These cells were associated with other granule cells in clusters similar to those described in the IGL (Fig. 3*C*).

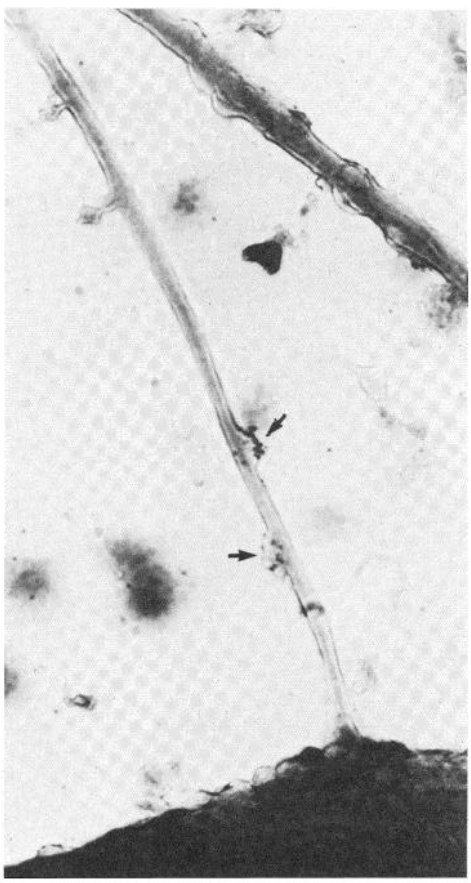

Figure 2. Autoradiogram of migrating granule cells (*arrows*) on cables which were labeled with [^{3}H]Thy prior to culturing.

As described above, migrating cells change their shape according to their activity. Figure 4 represents the distribution of labeled cells (one or two generations of granule cells) into the three categories defined above, as was determined over a period of 6 days in culture. After 2 days *in vitro* the majority (~60 to 80%) were in the running and intermediate state, whereas less than 10% fell into the category of standing cells. This proportion changed with increasing time in culture: whereas the percentage of running cells decreased, the percentage of standing cells increased until after 6 days *in vitro* when more than 60% of all labeled cells became postmigratory *in vivo*.

The evidence described above indicates that the timed sequence which regulates granule cell development is intrinsic to the granule cell.

Timed sequence of granule cell maturation in vitro reflects developmental state of granule cells in vivo. The results described above suggest that granule cells of a particular generation maintain their ability to migrate *in vitro* even 2 days after having entered the IGL *in vivo*. To demonstrate this, experiments have been designed to allow granule cell development *in vivo* and, at particular times, determine their state of maturation *in vitro*.

Mice were injected once with [^{3}H]Thy and maintained up to 6 days with their mother. Cerebellar cells were prepared for culture at 1 to 6 days after label and cultured for 3 days, and the percentage of labeled cells on cables was determined autoradiographically. The results are documented in Figure 5*A*. Five to 22% of cells on cables were labeled when prepared from mice which were injected with [^{3}H]Thy 1 day before culturing. This number increased to 32 to 43% when cultures were prepared from mice labeled 3 or 4 days before culturing. On the other hand, the percentage of labeled cells from mice injected with [^{3}H]Thy 5 to 6 days before culturing was reduced considerably to about 12 to 18% and 9 to 12%, respectively. The total number of cells remained constant throughout the culture period. These results support the evidence described above that granule cells maintain their ability to migrate for about 2 days after reaching the IGL *in vivo*.

It appears unlikely that the increase of labeled cells on cables or in reaggregates is due to cell division *in vitro*. When P3 cerebellar cells were cultured and pulse labeled for 3 hr with [^{3}H]Thy at various times *in vitro*, neither thymidine incorporation nor mitotic figures were observed in granule cell populations after 24 hr *in vitro* (not shown). Furthermore, the position and interaction of labeled cells in reaggregates and on cables indicate distinct stages of maturation. The results also indicate that reaggregates and cables provide the appropriate environment for particular stages in granule cell development.

Discussion

The experiments described are designed, first, to illustrate that the migrating cells on cables are granule cells and, second, to elucidate the time frame in which granule cells mature.

The time of origin and route of migration of immature granule cell neurons have been described in numerous studies (Miale and Sidman, 1961; Fujita, 1964, 1967; Fujita et al., 1966; Altman, 1982). Miale and Sidman (1961) showed that granule cells originate at the rhombic lip, spread as a thin layer across the external surface of the developing cerebellar anlage, and proliferate postnatally. Details on the timing of granule cell

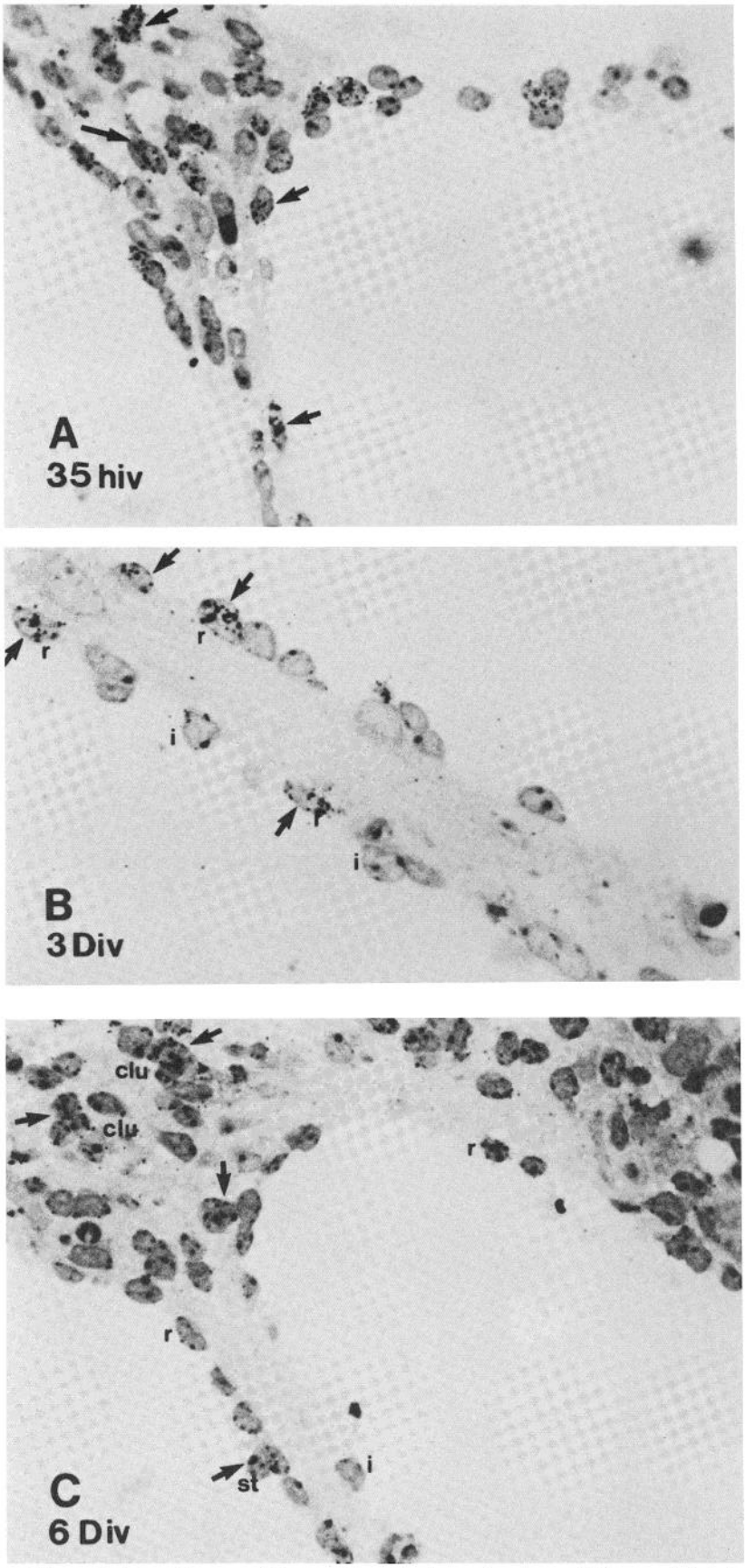

Figure 3. Autoradiogram of 1-μm sectioned microwell cultures. Cells were labeled with [^{3}H]Thy 4 hr before culturing. After 35 hr *in vitro* (*hiv*) (*A*), and 3 (*B*) and 6 (*C*) days *in vitro* (*Div*) cultures were fixed in 4% paraformaldehyde, embedded in Epon, and thin sectioned. *r*, running cells; *i*, intermediate cells; *st*, standing cells; *clu*, cluster of granule cells. Examples of labeled cells are marked with an *arrow*.

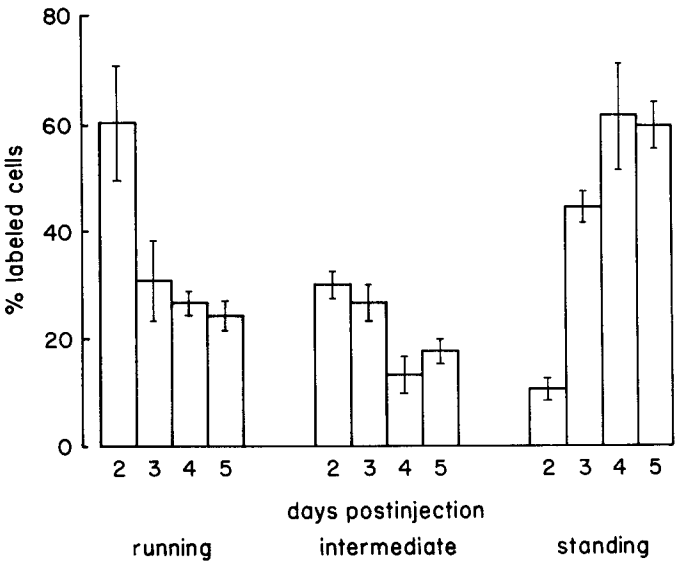

Figure 4. Distribution of [^{3}H]Thy-labeled cells among the three categories of migratory behavior (running, intermediate, and standing) after 2 to 5 days *in vitro*. A total number of 1500 cells was scored in six experiments, and the percentage of labeled to total granule cells was determined. The *error bars* represent the variation of the percentage of labeled to total cells in six experiments.

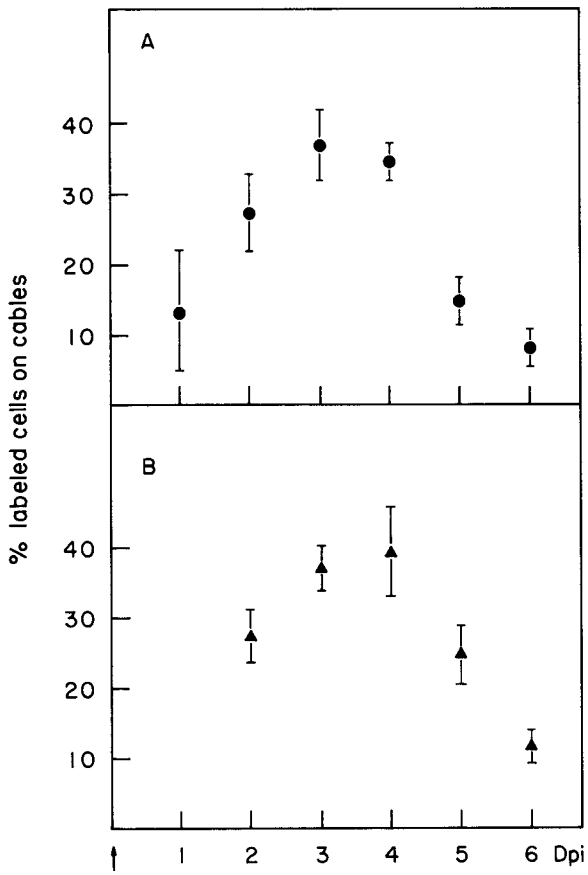

Figure 5. Number of labeled granule cells on cables (expressed as percentage of labeled to total cells). *A*, P3 mice were injected once with [^{3}H]Thy. In intervals of 1 to 6 days postinjection (*1* to *6 Dpi*) cerebellar cells were cultured for 3 days and the percentage of labeled cells was determined autoradiographically in six experiments. *B*, P3 mice were injected once with [^{3}H]Thy and after 4 hr were cultured for 6 days. In daily intervals the percentage of labeled cells was determined autoradiographically. The data were obtained from nine experiments. There were 1500 cells scored in *A* and *B*, respectively. The error bars demonstrate the variation of percentage of labeled cells in six experiments.

proliferation have been provided by the studies of Fujita in the mouse (Fujita, 1964, 1967; Fujita et al., 1966) and Altman for the rat (Altman, 1982). During the initial period of proliferation, all EGCs seem to be engaged in DNA synthesis and mitosis. Immediately after the S phase, [^{3}H]Thy-labeled cells move from a superficial position to occupy a postmitotic zone at the inner face of the EGL. In the mouse, labeled cells appear to wait in the postmitotic zone for 21 to 24 hr and then migrate into the deeper layers of the cortex. Other cells continue to transit the cell cycle and, after their final mitosis, seem to follow the same sequence to migrate into the IGL. According to Fujita et al. (1966), granule cells take less than 5 hr to migrate through the ML and are subsequently distributed throughout the IGL. The time frame which is required for granule cells to reach their final destination in the IGL is not yet known.

The results reported here suggest that the maturation of granule cells *in vitro* is comparable to that *in vivo*; pulse-labeled granule cells behaved *in vitro* as their *in vivo* counterparts did.

However, *in vitro*, the time frame of migration of granule cells appears to be prolonged. When cells were cultured before or during their migratory state *in vivo*, the majority of one cell generation labeled with [^{3}H]Thy continued to migrate for 3 to 4 days, which is 2 days longer than required to reach the IGL *in vivo* (Figs. 4 and 5). When, on the other hand, cells had entered the IGL 3 days before culturing, they did not migrate *in vitro*. These results suggest, first, that granule cells, once induced to migrate, maintain the ability to do so for 3 to 4 days before they either home in reaggregates or form clusters with other granule cells on cables. The results also suggest that granule cells might change their cell surface properties after their period of migration and that such changes are required in their forming clusters (Figs. 3 and 4). This behavior is not due to a change in culture conditions since unlabeled cells continue to migrate throughout the culture period.

Although the overall correlation of the time frame of granule cell maturation *in vivo* and *in vitro* reported here suggests that granule cell maturation is controlled intrinsically, certain extrinsic factors have to be considered. If cells are trypsinzed before culturing, 6 to 12 hr are required to rebuild their cell surface (E. Trenkner, unpublished observation). That is, under these conditions, cables are formed and cells can begin to migrate only after 28 to 30 hr in culture. Therefore, it is possible that this delay, built into the culture system, might be the explanation for the prolonged migration time *in vitro* as compared to *in vivo*. On the other hand, this delay did not affect cells which entered the IGL 3 days before culturing. In addition, the time sequence of granule cells which developed *in vivo* and were assayed *in vitro*, and that of granule cells developed *in vitro*, appeared to be the same.

In conclusion, we would like to postulate that the duration of granule cell migration in the cerebellum is controlled intrinsically. Secondly, the results suggest that granule cells continue to migrate after entering the IGL for a predetermined time unless they receive an external signal to stop.

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