Intracellular Ca^{2+} Fluctuations Modulate the Rate of Neuronal Migration

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Summary

Transient elevations of intracellular Ca^{2+} levels play critical roles in neuronal development, but such elevations have not been demonstrated in migrating neurons. Here, we show that the amplitude and frequency components of Ca^{2+} fluctuations are correlated positively with the rate of granule cell movement in cerebellar microexplant cultures. Moreover, depression of the amplitude and frequency components of Ca^{2+} fluctuations by blockade of Ca^{2+} influx across the plasma membrane results in a reversible retardation of cell movement. These results indicate that the combination of amplitude and frequency components of intracellular Ca^{2+} fluctuations may provide an intracellular signal controlling the rate of neuronal cell migration.

Introduction

In the developing mammalian brain, the migration of immature neurons from their birthplace to their final destinations is essential for the establishment of normal cytoarchitecture, synaptic connectivity, and function (Sidman and Rakic, 1973; Caviness and Rakic, 1978; Hatten, 1990; Rakic, 1990). In the cerebellar cortex, for example, granule cells migrate from the external granular layer where they are generated, crossing the expanding molecular layer to reach their final position in the internal granular layer (Ramon y Cajal, 1911; Miale and Sidman, 1961; Fujita et al., 1966). During migration, postmitotic granule neurons first extend their leading processes and then translocate their soma and surrounding cytoplasm within it (Rakic, 1971, 1981). Granule cell movement is characterized by alternations of short stationary phases with movement in a forward or backward direction (Komuro and Rakic, 1995). The net displacement of a cell depends on the duration and frequency of these phases as well as on the speed of movement (Komuro and Rakic, 1995). Electron microscopic analysis indicated that granule cell migration may be guided by interactions with the elongated processes of Bergmann glial cells (Rakic, 1971). Subsequent studies have supported the concept that functional interactions between postmitotic granule cells and Bergmann glial fibers are essential for neuronal migration (Rakic and Sidman, 1973a, 1973b; Rakic, 1976, 1981; Hatten et al., 1984, 1986; Hatten and Mason, 1990; Gao and Hatten, 1993).

In the past several years, it became apparent that this complex cellular process requires the orchestration of multiple molecular events, including the expression of specific cell adhesion and recognition molecules (Edelman, 1984; Antonicek et al., 1987; Edmondson et al., 1987; Pollerberg et al., 1987; Gloor et al., 1990; Fishell and Hatten, 1991; Cameron and Rakic, 1994; Rakic et al., 1994; Anton et al., 1996). In addition, rate of movement seems to be controlled by the activity of specific subtypes of neurotransmitter receptors and voltage-dependent ion channels (Komuro and Rakic, 1992, 1993; Rossi and Slater, 1993; Farrant et al., 1994; Rakic and Komuro, 1995; Slater and Rossi, 1996) as well as by the polymerization of contractile proteins (Rakic, 1985; Rivas and Hatten, 1995; Rakic et al., 1996). The rate of granule cell movement depends on both extracellular Ca^{2+} concentrations and Ca^{2+} influx through N-type Ca^{2+} channels (Komuro and Rakic, 1992) and the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor in cerebellar slice preparations (Komuro and Rakic, 1993). For example, lowering extracellular Ca^{2+} concentrations resulted in a graded decrease in the rate of granule cell migration, while elevation of extracellular Ca^{2+} concentrations slightly enhanced the rate of migration (Komuro and Rakic, 1992). Moreover, selective blockade of N-type Ca^{2+} channels or NMDA subtype of glutamate receptors (or both) by specific antagonists resulted in the curtailment of granule cell migration (Komuro and Rakic, 1992, 1993). Taken together, these results suggest that spontaneous fluctuations of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) may play a crucial role in regulating granule cell migration in the developing cerebellum. To determine whether changes in $[Ca^{2+}]_i$ are associated with the saltatory movement of migrating neurons, in the present study we examined intracellular Ca^{2+} dynamics during granule cell migration in cerebellar microexplant cultures.

Results

Cell Migration in Microexplant Cultures

In microexplant cultures of P2-P5 mouse cerebella, granule cells migrated extensively away from the center of the slice (Figure 1A). After 3 days in vitro, individual neurons have moved out up to a distance of 500 μm from the explants along radially oriented glial processes and bundles of neurites (Figure 1B). The majority of migrating cells were 6 μm–9 μm in diameter and assumed a characteristic bipolar shape with voluminous leading and thin trailing processes. Their overall shape and size were similar to migrating cerebellar granule cells described in vivo and in vitro (Rakic, 1971; Edmondson and Hatten, 1987; Gregory et al., 1988; Nagata and Nakatsuji, 1990). The rate of neuronal migration in this system ranged between 5 μm/hr–35 μm/hr ($n=85$), and is comparable with the movement of the granule cells in cerebellar slice preparations (Komuro and Rakic, 1995).

To examine spontaneous changes in $[Ca^{2+}]_i$ during neuronal migration, we loaded migrating cells in microexplant cultures with Ca^{2+} indicator dyes, Fluo-3 or Fura-Red (or both), and observed individual cells with a confocal microscope. We initially chose Fluo-3 as the Ca^{2+} indicator for this study because, among presently

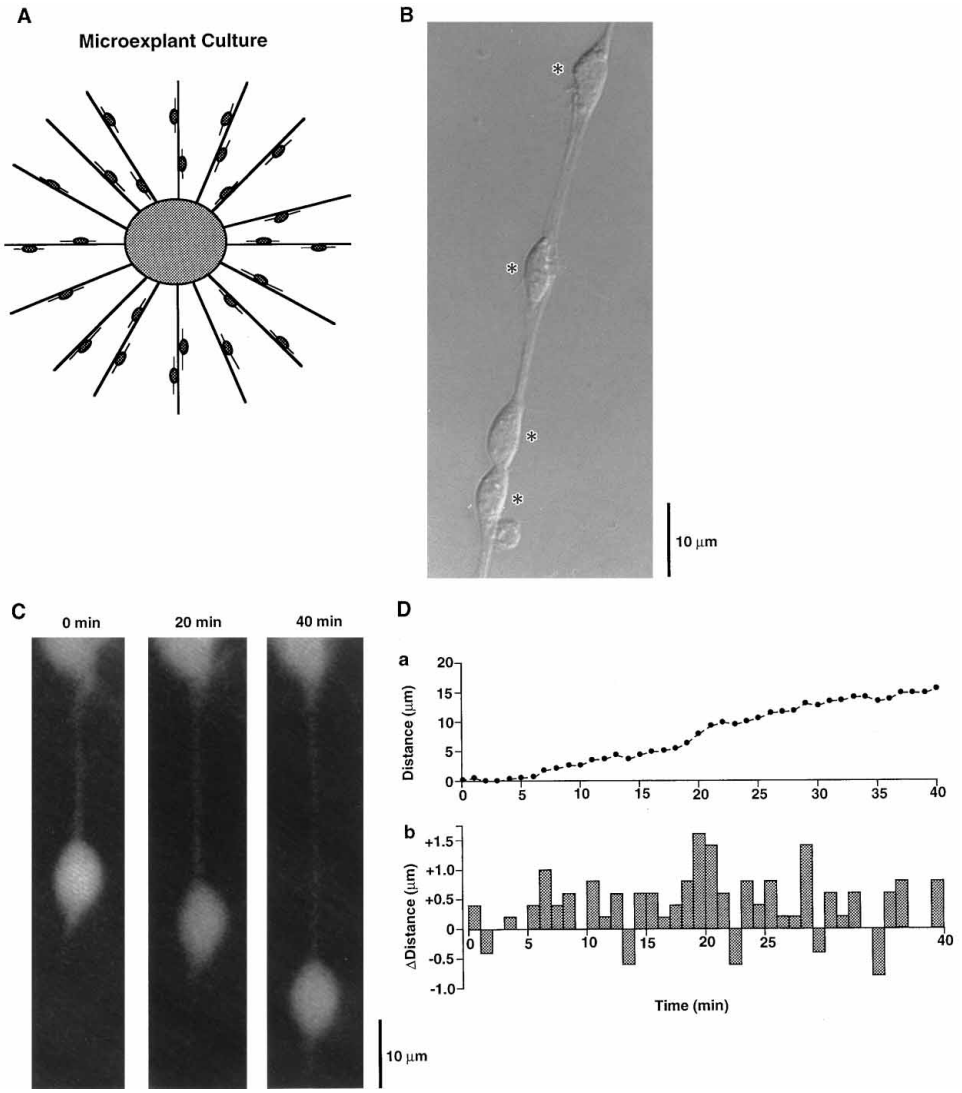

Figure 1. Granule Cell Migration in Cerebellar Microexplant Cultures

(A) Schematic diagram of cerebellar microexplant cultures with bipolar silhouettes of outwardly migrating neurons along bundles consisting of process of glial cells and neurites.

(B) Typical migrating neurons from a microexplant culture. Migrating neurons (marked by asterisks) in this assay display characteristic leading and trailing processes.

(C) A migrating granule cell loaded with 1 μM Fluo-3 shows change in fluorescence intensity over time indicated in minutes at the top of each photograph.

(D) Graphs showing the saltatory movement of the dye-loaded cell illustrated in (C). In (a), the distance traversed by the cell is plotted as a function of elapsed time. In (b), the direction and distance traversed by the cell during each minute of the testing period is shown. Positive values represent forward movement of the cell body, while negative values represent backward movement in this and in Figures 3, 4, and 7.

available indicators, it provides the largest optical signal per molecule and thus allows for detection of Ca^{2+} transients in the smallest possible cytoplasmic volumes (Minta et al., 1989). In addition, preliminary measurements with Fluo-3 showed the highest levels of fluorescence intensity in the nuclear region during movement of cell body (Figure 1C). Since our previous work demonstrated that an application of cell-permeant Ca^{2+} chelator, bis-(α-aminophenoxy)-ethane-N,N,N′,N′-tetra acetic acid, tetra (acetoxymethyl)-ester (BAPTA-AM) resulted in a graded decrease in the rate of granule cell migration in a dose-dependent manner (Komuro and Rakic, 1993), we decided to test for possible adverse effects of Fluo-3 application on cell movement. Cells loaded with 1 μM–3 μM Fluo-3 acetoxymethyl ester (AM) migrated at a rate ranging from 4 μm/hr–32 μm/hr with average values of 15 μm/hr ± 2 μm/hr (n=70), which is comparable with the movement of the granule cells in cerebellar slice preparations (Komuro and Rakic, 1992, 1993, 1995), and in dissociated cerebellar cocultures (Edmondson and Hatten, 1987; Fishell and Hatten, 1991). Furthermore, we observed that dye-loaded cells in microexplant culture exhibited saltatory movements (Figure 1D), similar to that described in cerebellar slice

preparations (Komuro and Rakic, 1995) and in dissociated cerebellar cocultures (Edmondson and Hatten, 1987). Even though we could not determine precisely the intracellular concentrations of Fluo-3 in the migrating cells, these results demonstrate that an application of 1 μM–3 μM Fluo-3 AM to the culture medium does not affect either the rate or the mode of cell movement.

Spontaneous Fluctuations of $[Ca^{2+}]_i$ during Migration

After establishing that the rate of cell movement was not adversely affected by addition of 1 μM–3 μM Fluo-3 AM to the culture medium, we examined whether $[Ca^{2+}]_i$ of migrating cells fluctuates spontaneously during movement of the cell body. First, continuous single-line scanning across the equator of migrating cell somata revealed a spontaneous small amplitude elevation of $[Ca^{2+}]_i$ with slopes rising and falling symmetrically (Figures 2A, 2B, and 2C). Among 60 migrating neurons examined by single-line scanning, $[Ca^{2+}]_i$ increased by an average of 14% ± 3% of baseline intensity, for an average duration of 1.3 min ± 0.4 min, and then decreased to baseline levels. Second, whole-cell frame images of $[Ca^{2+}]_i$ recorded at 1 min intervals also showed that $[Ca^{2+}]_i$ elevations were frequent and irregular during movement of the cell body (Figure 2D). Among 56 migrating neurons imaged for 1 hr each, transient elevations of $[Ca^{2+}]_i$ occurred 4 times per hour to 24 times per hour, with average frequencies of 13 per hour ± 3 per hour. This frequency is comparable with the cycle of saltatory movements observed in migrating granule cells in both slice preparations (Komuro and Rakic, 1995) and dissociated cell cultures (Edmondson and Hatten, 1987). These results suggest that migrating cells exhibit spontaneous and transient elevations of $[Ca^{2+}]_i$.

An alternative explanation for transient increases in Fluo-3 fluorescence intensity may be small changes in the thickness of migrating cells. To determine whether fluctuations of Fluo-3 fluorescence intensity reflect actual changes in $[Ca^{2+}]_i$ or fluctuations in the thickness of migrating cells, we conducted a series of ratiometric measurements of $[Ca^{2+}]_i$. Migrating cells were loaded with a mixture of two Ca^{2+} indicator dyes, Fluo-3 and Fura-Red. When excited at 514 nm upon Ca^{2+} binding, Fluo-3 exhibits an *increase* in the fluorescence intensity at 540 nm, while Fura-Red shows a *decrease* in the fluorescence intensity at 600 nm (Lipp and Niggli, 1993a, 1993b; Schild et al., 1994; Floto et al., 1995). Therefore, the ratio of Fluo-3 and Fura-Red intensity will accurately reflect changes in $[Ca^{2+}]_i$. In contrast, uniform changes of fluorescence intensity of both dyes would implicate artificial signals, including a change in cell thickness.

Simultaneous dual-emission images revealed that Fluo-3 and Fura-Red were colocalized within the soma of the migrating cell (Figure 3A). In contrast with the increase of fluorescence intensity obtained with Fluo-3, changes of fluorescence intensity measured with Fura-Red were characterized by transient decreases of the fluorescence intensity (Figure 3B). The relative fluorescence changes with Fura-Red were smaller than the corresponding Fluo-3 signals, but the onset of transient changes in both Fluo-3 and Fura-Red signals was similar (Figure 3B). Most importantly, the periodicity and time course, as well as the shape of $[Ca^{2+}]_i$ fluctuations obtained with Fluo-3/Fura-Red ratio, were identical to those obtained with the Fluo-3 alone (compare Figures 3B and 3C). Finally, the timing of each phase of Ca^{2+} fluctuations obtained with the Fluo-3/Fura-Red ratio correlates with the phases of saltatory movement of the migrating cell (compare Figures 3C and 3D). These results indicate that fluctuations of fluorescence intensity detected with Fluo-3 accurately reflect changes in $[Ca^{2+}]_i$ in migrating cells during movement of the cell body. Therefore, we used the Fluo-3 alone in the remaining experiments.

Temporal Correlation between Cell Movement and Ca^{2+} Fluctuations

To examine whether the spontaneous fluctuations of $[Ca^{2+}]_i$ play a role in cell movement, the relationship between the direction and distance traversed by the cell body and the time course of intracellular Ca^{2+} changes was determined. During a recording period of 45 min, the migrating cell typically exhibited 8 cycles of saltatory movement and a corresponding number of transient elevations of $[Ca^{2+}]_i$ (Figures 4A and 4B). Furthermore, each phase of saltatory movement of the migrating cell correlated temporally with each phase of spontaneous Ca^{2+} fluctuations. For example, the cell illustrated in Figures 4A and 4B exhibited forward movement during the phase of transient elevations of $[Ca^{2+}]_i$. In particular, at the peak of each Ca^{2+} elevation, the distance traversed by the cell body was the largest. In contrast, at each trough of Ca^{2+} fluctuations, the cell remained stationary or exhibited small backward movement.

Correlation between the Rate of Cell Movement and the Amplitude/Frequency of Ca^{2+} Fluctuations

To determine the relationship between $[Ca^{2+}]_i$ fluctuations and cell motility, we examined the amplitude and frequency of Ca^{2+} elevations as a function of the rate of single-cell movement. In spite of considerable variations in intracellular Ca^{2+} elevations in individual cells, there was a clear positive correlation between the rate of cell movement and both the amplitude and frequency components of Ca^{2+} fluctuations (Figures 4C and 4D). The correlation coefficient between the rate of cell movement and the amplitude of Ca^{2+} fluctuations was 0.74, and the value between the rate of cell movement and the frequency of Ca^{2+} fluctuations was 0.83. For example, fast-moving cells exhibited a larger amplitude and higher frequency of Ca^{2+} fluctuations than slow-moving cells (Figures 4C and 4D). Furthermore, cells that displayed higher amplitude Ca^{2+} fluctuations demonstrated more frequent changes in $[Ca^{2+}]_i$ than cells that displayed lower amplitudes. Taken together, these results suggest that both the amplitude and frequency components of Ca^{2+} fluctuations may be involved in the rate of cell movement.

To test whether changes in the amplitude and frequency of intracellular Ca^{2+} fluctuations affect the rate of cell migration, we altered these parameters either by changing Ca^{2+} influx through the plasma membrane or by decreasing Ca^{2+} release from intracellular Ca^{2+} stores. Reduction of Ca^{2+} influx by lowering extracellular

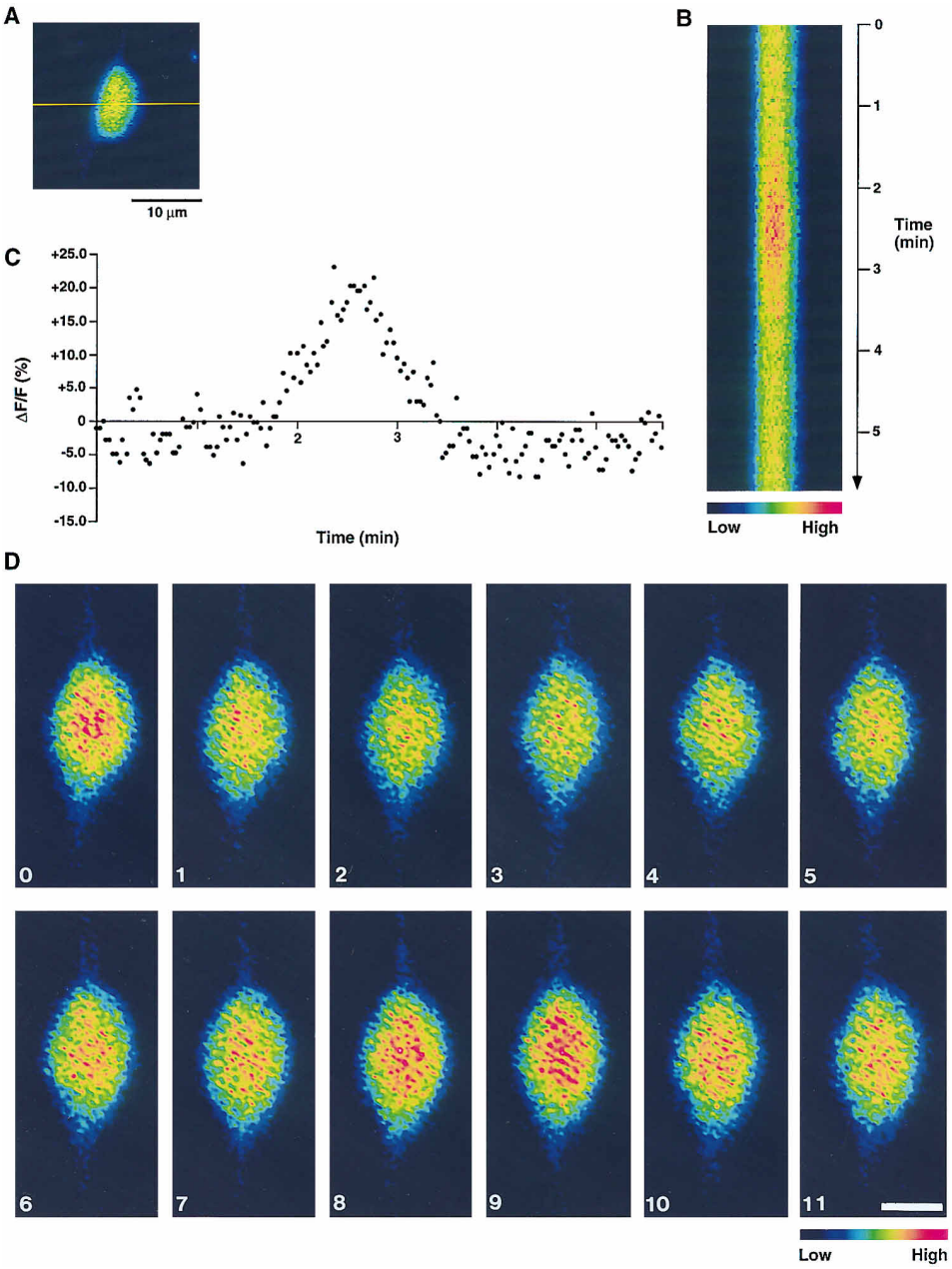

Figure 2. Spontaneous Ca^{2+} Elevations in Migrating Granule Cells

(A) Pseudocolor frame image of $[Ca^{2+}]_i$ in the migrating cell loaded with 1 μM Fluo-3. Yellow line indicates the equator of cell soma.

(B) Pseudocolor line-scan image of $[Ca^{2+}]_i$ obtained by scanning the beam at the relative position indicated by the yellow horizontal line through the cell soma in (A). To measure changes in $[Ca^{2+}]_i$ over time at the relative position of the cell body of migrating cells, a single line was scanned at a fixed point every 2 s, for a period of 6 min. Sequential intensities of the fluorescence signal are displayed from top to bottom.

(C) Time course of intracellular Ca^{2+} change in the cell illustrated in (B). Upward deflections represent elevations of $[Ca^{2+}]_i$ and downward deflections indicate decreases of $[Ca^{2+}]_i$ in this and in Figures 4 and 7. The cell body moved forward or backward (or both) at a total distance ranging from 0.5 μm–3.0 μm during the 6-min line-scan recording. Initial line-scan point was fixed at the center of the cell body. The fluorescence intensity in the center area of the cell body, which was an ~4 μm–5 μm-diameter oval, is uniform as seen in Figures 2A, 2D, and 3A. Therefore, even if the position of the fixed line-scan shifts within the center area of the cell body, owing to cell movement during recording without a spontaneous fluctuation of $[Ca^{2+}]_i$, the fluorescence intensity remains constant. Furthermore, the fluorescence intensity at the initial line-scan point, a center of the cell body, was the highest in the migrating cell. Thus, if the fixed line-scan position shifts beyond the center area to the front or rear part of the cell body without spontaneous changes of $[Ca^{2+}]_i$, the fluorescence intensity should decrease monotonically. Therefore, we assume that any changes of relative position between the cell body and line-scan point do not contribute substantially to the recorded spontaneous Ca^{2+} elevation.

(D) Pseudocolor frame images of a migrating cell exhibiting spontaneous elevations in $[Ca^{2+}]_i$. Although the occurrence and amplitude of intracellular Ca^{2+} elevations were not regular, the migrating cell exhibited spontaneous multiple elevations of $[Ca^{2+}]_i$ during somal translocation. Numerals at the bottom of each photograph indicate time in minutes. Scale bar, 5 μm.

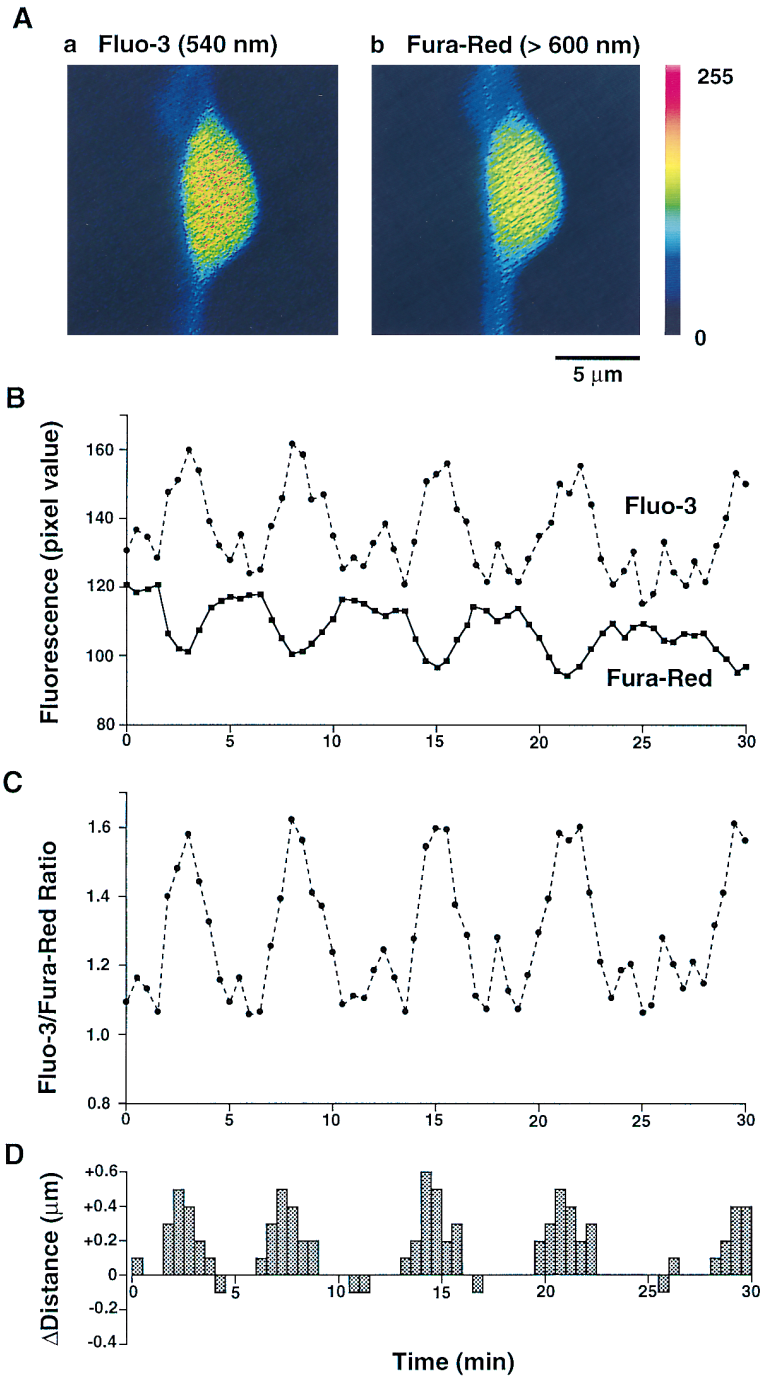

Figure 3. Ratiometric Measurements of Spontaneous Intracellular Ca^{2+} Fluctuations in Migrating Neurons

(A) Distribution of Fluo-3 and Fura-Red in the migrating cell. The cellular distribution of both dyes was determined by using pseudocolor representations of fluorescence intensity pixel values taken at 540 nm ± 15 nm for Fluo-3 and >600 nm for Fura-Red. Fluorescence intensity is shown on a linear color scale (range 0–255).

(B) Changes of fluorescence intensity of Fluo-3 and Fura-Red taken from the soma of the cell shown in (A). Both fluorescence intensities were simultaneously measured with the dual-emission mode of a confocal microscope every 30 s. Upward deflections in Fluo-3 signals represent elevations of $[Ca^{2+}]_i$ and downward deflections indicate decreases of $[Ca^{2+}]_i$. In contrast, upward deflections in Fura-Red signals represent decreases of $[Ca^{2+}]_i$ and downward deflections indicate elevations of $[Ca^{2+}]_i$. Fluo-3 fluorescence intensity: dotted line. Fura-Red florescence intensity: solid line.

(C) Time course of changes in Fluo-3/Fura-Red ratio signal. Upward deflections in Fluo-3/Fura-Red ratio signals represent elevations of $[Ca^{2+}]_i$ and downward deflections indicate decreases of $[Ca^{2+}]_i$.

(D) The direction and distance traversed by the same cell during each 30 s of the testing period.

Ca^{2+} concentrations from 1.8 mM to 0.1 mM resulted in a 68% decrease in the amplitude of spontaneous Ca^{2+} elevations (Figure 5A). Moreover, an addition of a voltage-dependent N-type Ca^{2+} channel antagonist (1 μM ω-conotoxin [ω-CgTx]) or an NMDA receptor antagonist (100 μM D-2-amino-5-phosphonopentanoic acid) [D-AP5]) to the medium resulted in a decrease in the amplitude of spontaneous Ca^{2+} elevations to 45% and 49% of control value, respectively (Figure 5A). This reduction was linearly related to the rate of cell movement (Figure 5A). The reduced Ca^{2+} concentrations and addition of ω-CgTx and D-AP5 also reduced the frequency of Ca^{2+} fluctuations to 62%, 85%, and 74% of control value, respectively (Figure 5B).

To examine whether intracellular Ca^{2+} stores contribute to the observed Ca^{2+} fluctuations during cell migration, we applied 1 μM thapsigargin to the culture medium. Thapsigargin inhibits Ca^{2+}–ATPase and subsequently results in depletion of intracellular Ca^{2+} stores (Lankford et al., 1995; Shmigol et al., 1995). Furthermore, at higher concentrations, this compound may have additional effects, such as the reduction of voltage-dependent Ca^{2+} channel activity observed in pituitary cells (Nelson et al., 1994). Addition of thapsigargin to the

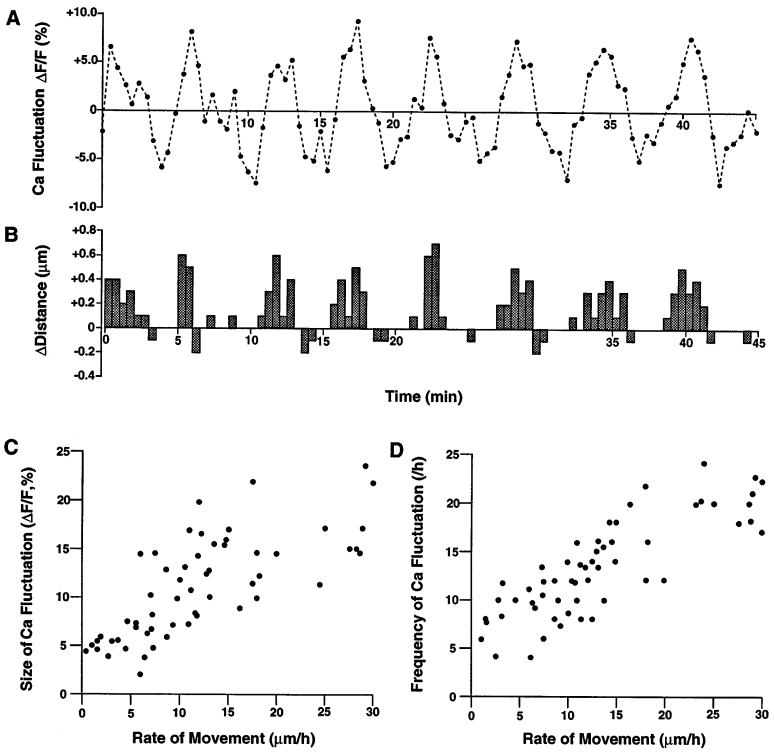

Figure 4. Relationship between Ca^{2+} Fluctuations and Cell Movement

(A) and (B) Temporal correlation between individual Ca^{2+} fluctuations and individual cell movement during migration. (A) Example of the time course of intracellular Ca^{2+} changes in a migrating cell.

(B) The direction and distance traversed by same cell during each minute of the testing period. Frame images of migrating cells loaded with 1 μM Fluo-3 were recorded every 30 s for 45 min.

(C) and (D) Relationship between the amplitude component of Ca^{2+} fluctuations and the rate of cell movement (C), and between the frequency component of Ca^{2+} fluctuations and the rate of cell movement (D). Each point displays either the size or frequency of Ca^{2+} fluctuations in relation to rate of cell movement of a single neuron.

culture medium decreased the amplitude of Ca^{2+} elevations to 90% of control value and slowed cell movement to 78% of control value without significant effect on the frequency of Ca^{2+} elevations (Figures 5A and 5B). These results suggest that the release of Ca^{2+} from intracellular stores contributes to the Ca^{2+} fluctuations observed during the cell movement.

Since average $[Ca^{2+}]_i$, independent of Ca^{2+} fluctuations, may also affect the rate of cell movement, we increased $[Ca^{2+}]_i$ by adding a Ca^{2+} ionophore, ionomycin (Lankford and Letourneau, 1989; Ivins et al., 1991) and examined the effect on cell movement. Addition of ionomycin (100 nM) to the medium increased the average intracellular Ca^{2+} level ($F_{1/2}$) (which was calculated as the Ca^{2+} level midway between peak and trough of Ca^{2+} fluctuations, from 92 nM ± 30 nM to 250 nM ± 60 nM) (n=28), but did not affect significantly the amplitude and frequency of Ca^{2+} fluctuations (Figures 6A, 6B, and 6C). Importantly, ionomycin-induced elevations of average $[Ca^{2+}]_i$, without a corresponding change in the amplitude and frequency of Ca^{2+} fluctuations, failed to affect the rate of cell movement (Figure 6D).

The relationship between intracellular Ca^{2+} fluctuations and cell migration was examined by blocking Ca^{2+} fluctuations in migrating cells that showed elevated $[Ca^{2+}]_i$. The addition of ω-CgTx (1μM), D-AP5 (100 μM), thapsigargin (1 μM), and ionomycin (50 nM) to the medium increased the average $[Ca^{2+}]_i$ from 101 nM ± 26

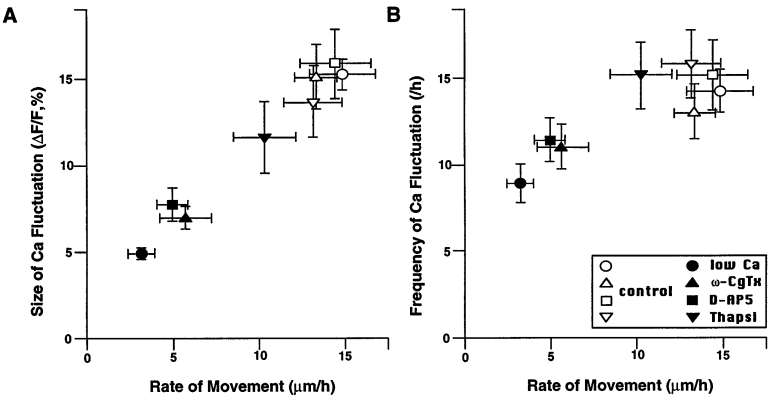

Figure 5. Reduction of Ca^{2+} Influx and Ca^{2+} Release Affects Both Ca^{2+} Fluctuations and the Rate of Cell Movement

Effect of blockade of Ca^{2+} influx and Ca^{2+} release from intracellular Ca^{2+} stores on the relationship between the amplitude of Ca^{2+} fluctuations and the rate of cell movement (A), and between the frequency of Ca^{2+} fluctuations and the rate of cell movement (B). Ca^{2+} influx across the plasma membrane was reduced by a decrease in extracellular Ca^{2+} concentrations from 1.8 mM to 0.1 mM, or by addition of 1 μM ω-CgTx or 100 μM D-AP5. Ca^{2+} release from intracellular Ca^{2+} stores was reduced by an application of 1 μM thapsigargin (Thapsi). Each agent was added to culture medium in separate experiments after 30 min of observation. Images were recorded every minute for a total of 60 min, including the period before and after the addition of each agent. Each experimental group (indicated by filled symbols) should be compared with the appropriate control (indicated by open symbols). Each value is the average obtained from 10 migrating cells. Bar, SD.

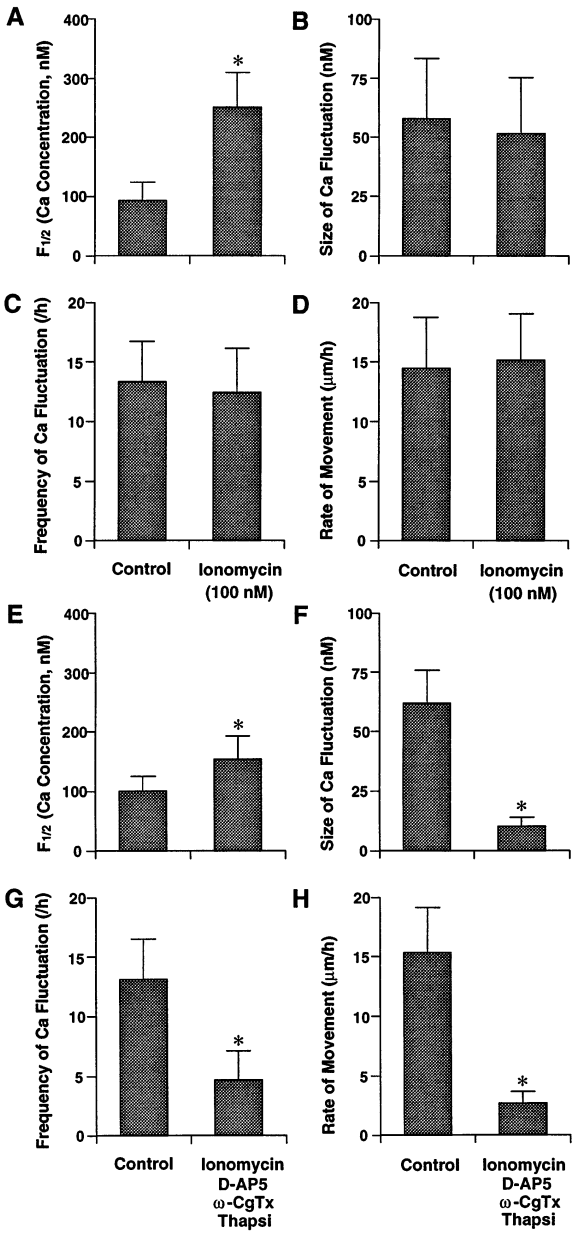

Figure 6. Changes in Average $[Ca^{2+}]_i$ Do Not Affect the Rate of Cell Movement

(A)–(D) Effect of application of the Ca^{2+} ionophore, ionomycin, on (A) the average Ca^{2+} level ($F_{1/2}$), (B) the size of Ca^{2+} fluctuations, (C) the frequency of Ca^{2+} fluctuations, and (D) the rate of cell movement in migrating cells. Ionomycin (100 nM) was added to culture medium after 30 min of observation.

(E–H) Effect of blockade of spontaneous Ca^{2+} fluctuations and tonic elevations in $[Ca^{2+}]_i$ on the rate of cell movement.

(E) The average Ca^{2+} level ($F_{1/2}$).

(F) The size of Ca^{2+} fluctuations.

(G) The frequency of Ca^{2+} fluctuations.

(H) The rate of cell movement. ω-CgTx (1 μM), D-AP5 (100 μM), thapsigargin (1 μM), and ionomycin (50 nM) were added to culture medium after 30 min of observation. Images were recorded every minute for a total of 60 min including the period before and after the addition of these compounds. The average Ca^{2+} level ($F_{1/2}$) indicates the Ca^{2+} level midway between peak and trough of Ca^{2+} fluctuations. Each column represents the average obtained from 28 migrating cells (A–D) and 10 migrating cells (E–H). Asterisks indicate statistical significance ($p < 0.01$). Bar, SD.

nM to 154 nM ± 39 nM (n=10) (Figure 6E). The size of these tonic elevations of average $[Ca^{2+}]_i$ was approximately similar to the elevations of Ca^{2+} levels seen during spontaneous Ca^{2+} fluctuations (Figures 6E and 6F). However, the application of these compounds decreased both the amplitude (to 16% of control value) and frequency (to 36% of control value) of Ca^{2+} fluctuations (Figures 6F and 6G). Importantly, direct blockade of spontaneous Ca^{2+} fluctuations with concomitant tonic elevations of average $[Ca^{2+}]_i$ in the migrating cells resulted in a significant decrease in the rate of cell movement to 18% of control value (Figure 6H). However, since the cumulative cytotoxic effects of ω-CgTx, D-AP5, thapsigargin, and ionomycin are unknown, we cannot exclude the possibility that such potential cytotoxic effects may contribute to the observed decrease in the rate of cell movement.

To examine whether the transient elevations in $[Ca^{2+}]_i$ can modify the rate of cell migration, $[Ca^{2+}]_i$ was repeatedly elevated by short (3 min) pulses of high KCl delivered every 14 min. Each application of 30 mM KCl resulted in large elevations in $[Ca^{2+}]_i$ and concomitant increases in the forward movement of migrating cells (Figures 7A and 7B). Furthermore, the application of high KCl significantly increased the distance traveled by the cell during the accelerated forward movement. For example, the average distance traveled by the cell during each 30 s of forward movement was 0.3 μm at control levels, while the distance during the application of high KCl increased to 0.6 μm.

Taken together, present results indicate that changes in the amplitude and frequency of Ca^{2+} fluctuations associated with Ca^{2+} influx affect directly the rate of cell movement. Although Ca^{2+} release from intracellular Ca^{2+} stores may participate in the regulation of spontaneous Ca^{2+} fluctuations, Ca^{2+} influx through the plasma membrane seems to be the more potent mode of regulation of cell movement.

Discussion

The present study reveals that migrating cells exhibit spontaneous and transient elevations of $[Ca^{2+}]_i$ during their saltatory movement. Real-time recording of $[Ca^{2+}]_i$ suggests that individual fluctuations of $[Ca^{2+}]_i$ in migrating neurons may control different phases of their saltatory movement. Moreover, we found a positive correlation between the rate of cell movement and the amplitude and frequency components of Ca^{2+} fluctuations. However, the rate of neuronal migration did not depend on the average $[Ca^{2+}]_i$ in their soma.

In microexplant cultures of early postnatal mouse cerebella, neurons migrated out radially from the explants, along the elongated processes of glial cells or bundles of neurites (or both) on poly-L-lysine/laminin substrate. The majority (>90%) of migrating neurons in such cultures are granule cells, while the rest are assumed to

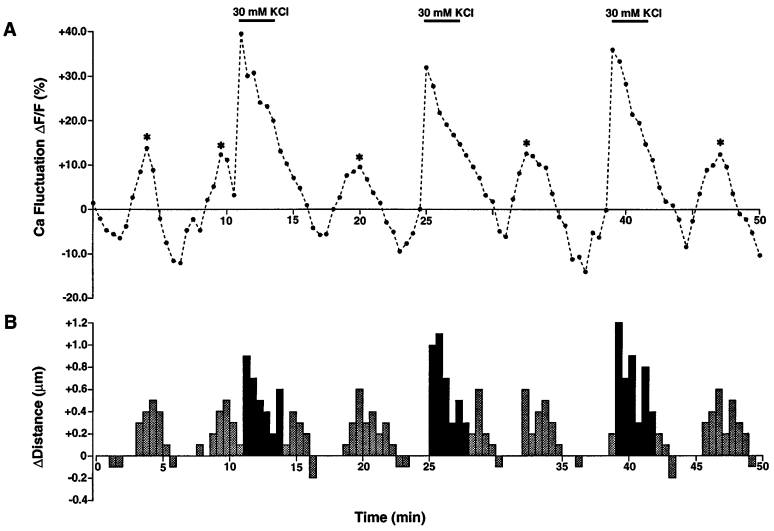

Figure 7. Effect of Transient Elevations of $[Ca^{2+}]_i$ on the Saltatory Movement of Migrating Cells

(A) Example of the time course of intracellular Ca^{2+} changes in a migrating cell loaded with 1 μM Fluo-3.

(B) The direction and distance traversed by the same cell during each 30 s of the testing period. $[Ca^{2+}]_i$ in the migrating cell were repeatedly elevated by application of short (3 min) pulses of 30 mM KCl. Each asterisk indicates the peak of spontaneous Ca^{2+} fluctuations. Filled columns represent the direction and distance traversed by the cell during the application of high KCl, while stippled columns represent spontaneous movements of the same cell.

be either basket cells or stellate cells (Nagata and Nakatsuji, 1990). The cytological and behavioral characteristics of migrating cells examined in the present study are quite similar to those of migrating cerebellar granule cells described in previous in vitro and in vivo studies (Rakic, 1971; Edmondson and Hatten, 1987; Komuro and Rakic, 1995). These cells have a typical bipolar shape and voluminous leading and thin trailing processes. During their movement, cell extended their leading process by rapid protrusion and retraction of the growing tip.

Our results suggest that spontaneous elevations of $[Ca^{2+}]_i$ may modulate significantly migratory behavior of postmitotic neurons. Previous studies showed that transient elevations of $[Ca^{2+}]_i$ are essential for initiating and maintaining the movement of cells ranging from fibroblast to immature glial cells (Newgreen and Gooday, 1985; Sawyer et al., 1985; Jaconi et al., 1991; Moran, 1991; Brundage et al., 1993; Anton et al., 1995). Thus, an increase in the rate of Schwann cell migration induced by application of antibodies to the cell surface glycoprotein, CD9, was correlated with a rise in $[Ca^{2+}]_i$ (Anton et al., 1995). Likewise, migrating human neutrophils exhibit multiple increases and decreases in $[Ca^{2+}]_i$ (Marks and Maxfield, 1990). Buffering of $[Ca^{2+}]_i$ by an addition of cell-permeant Ca^{2+} chelator BAPTA-AM or removal of extracellular Ca^{2+} blocks transient increases in $[Ca^{2+}]_i$ in neutrophils and, thus, reduces or inhibits cell migration (Marks and Maxfield, 1990).

It is not well understood how transient elevations of $[Ca^{2+}]_i$ control the cell motility. One possibility is that fluctuations of $[Ca^{2+}]_i$ regulate the dynamic assembly and disassembly of cytoskeletal elements required for the operation of a force-generating mechanism involved in cell movement (Rakic et al., 1996). Furthermore, elevation of $[Ca^{2+}]_i$ in neutrophils leads to the disruption of specific sites of attachment to an adhesive substratum (Marks and Maxfield, 1990). In analogy, changes in $[Ca^{2+}]_i$ in migrating neurons may modulate the repetitive formation and elimination of binding sites between migrating neurons and their migratory substrates. Intracellular Ca^{2+} may control conformational changes of cell adhesion molecules, such as integrin families, which are expressed on the plasma membrane of migrating neurons (Fishell and Hatten, 1991; Hynes, 1992; Clark and Brugge, 1995; Lawson and Maxfield, 1995).

Cellular mechanisms underlying the generation of Ca^{2+} fluctuations observed in migrating neurons are similar to those of growth cones, but there are also noticeable differences (Kater and Mills, 1991). For example, our results demonstrate that the rate of cell migration is positively correlated with the amplitude and frequency components of Ca^{2+} fluctuations. In contrast, spontaneous or experimentally induced Ca^{2+} spikes (or both) in growth cone slow the neurite migration (Gomez et al., 1995). Neurite outgrowth is significantly reduced immediately and transiently following Ca^{2+} spikes (Gomez et al., 1995). It is important to emphasize that transient elevations of $[Ca^{2+}]_i$ in growth cones do not synchronize with the elevation of $[Ca^{2+}]_i$ in cell bodies of differentiating neurons (Gomez et al., 1995). Moreover, $[Ca^{2+}]_i$ in growth cones fluctuates more frequently and widely than $[Ca^{2+}]_i$ in cell bodies in differentiating neurons (Gomez et al., 1995). However, the major difference between these two cellular events is translocation of cell nucleus and surrounding cytoplasm in the migrating neurons. This translocation profoundly may depend on the assembly and disassembly of microtubules (Gregory et al., 1988; Rakic et al., 1996), whereas the motility of growth cones depends largely on the assembly and disassembly of actin filaments (Forscher et al., 1992). Such differences may explain, in part, difference between roles of Ca^{2+} fluctuations in neuronal cell migration and neurite outgrowth.

In addition to the regulation of cell motility, spontaneous Ca^{2+} fluctuations have been shown to be essential for maturation of voltage-gated ion channels and the normal appearance of neurotransmitter in differentiating neurons (Spitzer, 1994; Gu and Spitzer, 1995). Therefore, Ca^{2+} fluctuations in migrating neurons may be a prerequisite for the selective expression and synthesis of cytoplasmic and membrane proteins essential for somatic translocation. The present results support the hypothesis that the combination of amplitude and frequency

components of Ca^{2+} fluctuations holds a key for the multiple cellular and molecular events involved in neuronal cell migration. The present results strengthen the concept that multiple cellular and molecular mechanisms are engaged in granule cell migration (Rakic et al., 1994).

Experimental Procedures

Substrata

Coating with poly-L-lysine was made on glass coverslips (25 mm in diameter) (Macalaster Bicknell Corporation). Sterile coverslips, immersed in a 100 μg/ml poly-L-lysine hydrobromide (molecular weight >300,000) (Sigma Chemical Company, St. Louis, MO) solution, were dried under a sterile air flow, rinsed with distilled water, and used as the poly-L-lysine substratum. About 100 μl of a solution of laminin (20 μg/ml) (Sigma) were applied to the dried poly-L-lysine substratum for 1 hr–2 hr at 37°C, rinsed three times with culture medium, and used immediately as the poly-L-lysine/laminin substrate.

Cell Culture

Postnatal (P) day 2 to day 5 mice (CD-1) were killed by decapitation, in accordance with institutional guidelines. Cerebella were removed quickly from the skull and placed in cold (5°C) Hanks' balanced salt solution (40 mM glucose, 2.5 mM Hepes) that was oxygenated with 95% O_2, 5% CO_2 to a final pH of 6.7. Although the pH of this medium is slightly lower than that used in previous cerebellar slice experiments (Komuro and Rakic, 1995), we observed no significant difference in cell viability or the rate of cell movement. Cerebella were freed from meninges and choroid plexus. Then slices were made with a surgical blade, from which white matter and deep cerebellar nuclei were removed. Rectangular pieces (100 μm–200 μm) were dissected out from the remaining tissue, which mainly consisted of the cerebellar gray matter, using a surgical blade. Such prepared microexplants were rinsed with the culture medium and placed evenly on the poly-L-lysine/laminin-coated glass coverslips (5–10 microexplants/coverslips) with 100 μl of the culture medium. One or two hours after the plating, each coverslip was transferred into a Petri dish (35 mm in diameter) (Corning Glass Works, Corning, NY), added with 1 ml of the culture medium, and put in a CO_2 incubator for 2 days to 3 days (37°C, 95% air, 5% CO_2). The incubation medium consisted of minimum essential medium (GIBCO) supplemented with 10% fetal calf serum, 30 mM glucose, 1.8 mM glutamine, 24 mM $NaHCO_3$, 90 U/ml penicillin, and 90 μg/ml streptomycin.

Loading of Ca^{2+} Indicator Dyes

The $[Ca^{2+}]_i$ in migrating neurons was monitored using the Ca^{2+} indicator dye Fluo-3 (Molecular Probes, Eugene, OR). After 2 days to 3 days in vitro (37°C, 95% air, 5% CO_2), cells were loaded with cell-permeant AM form of 1 μM–3 μM Fluo-3 diluted in serum-free defined culture medium for 30 min at 37°C. The cells were subsequently washed five times with serum-free defined culture medium, and the dye was allowed to deesterify for an additional 30 min–60 min in the CO_2 incubator. In some experiments, intracellular Ca^{2+} concentrations in migrating cells were estimated using a ratiometric method. For ratiometric Ca^{2+} measurements, cells were coloaded with cell-permeant AM form of 3 μM Fluo-3 and 4 μM Fura-Red (Molecular Probes) diluted in serum-free defined culture medium for 30 min at 37°C. The defined culture medium used for loading of Ca^{2+} indicator dyes and Ca^{2+} measurements consisted of minimum essential medium (GIBCO) supplemented with 30 mM glucose, 1.8 mM glutamine, 24 mM $NaHCO_3$, 90 U/ml penicillin and 90 μg/ml streptomycin.

Ca^{2+} Measurements

Coverslips were transferred into the chamber of a micro-incubator (PDMI-2) (Medical System Corporation) attached to the stage of an inverted microscope (IMT-2) (Olympus Corporation). Chamber temperature was kept at 37.0°C ± 0.5°C using temperature controller (TC-202) (Medical System Corp.), and the cells were provided with a constant gas flow (95% O_2, 5% CO_2). A laser scanning confocal microscope (BioRad Laboratories, Richmond, CA) (MRC-600) was used to examine the rate of cell movement and the intracellular Ca^{2+} level. The migrating cells loaded with Fluo-3 were illuminated with 488-nm wavelength light from an argon laser through the epifluorescence inverted microscope equipped with a ×40 water immersion objective (Zeiss, numerical aperture 0.75) or a ×60 oil immersion objective (Olympus, numerical aperture 1.40). Images of the migrating cells in a single focal plane were collected with laserscans every 0.5 s–60 s for up to 100 min, and recorded on an optical disk recorder (TQ-3031) (Panasonic) or an optical drive (comos 600, Racet). Changes in fluorescence intensity of each neuron were normalized to its baseline fluorescent intensity. Images of loaded cells also were carried out with a charge-coupled device color camera (NC-15) (NEC) and their position recorded on an optical disk recorder. Statistical significance between experimental groups was tested by Student's t test.

To visualize changes in $[Ca^{2+}]_i$ in migrating neurons in the microexplant cultures, we used the laser scanning confocal microscope with Ca^{2+} indicator dye Fluo-3. One problem in our experiment was that if the cell body shape of migrating neurons changes during their movement and migrating neurons move out under or above the sectioning plane of confocal microscope, change of intensity of Fluo-3 may not directly relate to the changes of $[Ca^{2+}]_i$. To minimize this problem, we used a larger aperture size of light detector of confocal microscope in order to increase the thickness of sectioning plane.

We estimated $[Ca^{2+}]_i$ in migrating cells in some experiments using a previously described procedure (Kao et al., 1989). The following equation was used to determine the free intracellular Ca^{2+} concentration: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the Ca^{2+}–Fluo-3 dissociation constant (400 nM), F is the measured fluorescence intensity, F_{min} is the florescence intensity in the absence of Ca^{2+}, and F_{max} is the fluorescence intensity of the Ca^{2+}-saturated dye. F_{max} is calculated as $(F_{Mn} - F_{bkg})/0.2 + F_{bkg}$, where F_{Mn} is the fluorescence intensity with ionomycin (5 μM) and $MnSO_4$ (2 mM) and F_{bkg} is the fluorescence intensity with ionomycin (5 μM) and $MnSO_4$ (2 mM) after lysis using 40 μM digitonin. F_{min} is calculated as $(F_{max} - F_{bkg})/40 + F_{bkg}$, since metal-free Fluo-3 has 1/40 the fluorescence of the Ca^{2+} complex. Like other calibration methods in which cytosolic Ca^{2+} is not actually clamped to known levels in situ, this procedure assumes that dye in cells behaves similarly to dye in vitro, particularly with regard to both the K_d values for Ca^{2+} and the relative fluorescence efficiencies for the Ca^{2+} and heavy metal complex with respect to the uncomplexed species. Importantly, changes in the spatial distribution of Fluo-3 in moving cells preclude a precise estimation of absolute $[Ca^{2+}]_i$.

To obtain ratiometric measurements of intracellular Ca^{2+} concentrations in the migrating cells, fluorescence emission from the two Ca^{2+} indicators, Fluo-3 and Fura-Red, was recorded simultaneously in the dual-emission mode of a MRC-600 laser scanning confocal microscope. The 514-nm line of the argon laser was used to excite both fluorophores while fluorescence emission was detected simultaneously at 540 nm ± 15 nm (Fluo-3) and >600 nm (Fura-Red). Dual-emission images of the migrating cells loaded with Fluo-3 and Fura-Red in a single focal plane were collected with laserscans every 30 s for up to 60 min, and recorded on an optical disk recorder or an optical drive. Fluo-3/Fura-Red ratio images were calculated by dividing pixel values of Fluo-3 fluorescence intensity by pixel values of Fura-Red fluorescence intensity.

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