Regulation of Dendritic Spine Motility in Cultured Hippocampal Neurons

Eduard Korkotian and Menahem Segal

Department of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel

Regulation of dendritic spine motility was studied in dissociated cultures of the rat and mouse hippocampus, using green fluorescent protein-labeled neurons or neurons loaded with the calcium-sensitive dye Oregon Green-1. Cells were time-lapse-photographed on a confocal laser-scanning microscope at high resolution to detect movements as well as spontaneous fluctuations of intracellular calcium concentrations in their dendritic spines. Active presynaptic terminals attached to the spines were labeled with FM4-64, which marks a subset of synaptophysin-labeled terminals. Dendritic spines were highly motile in young, 4- to 7-d-old cells. At this age, neurons had little spontaneous calcium fluctuation or FM4-64 labeling. Within 2–3 weeks in culture, dendritic spines were much less

motile, they were associated with active presynaptic terminals, and they expressed high rates of spontaneous calcium fluctuations. Irrespective of age, and even on the same dendrite, there was an inverse relationship between spine motility and presence of FM4-64-labeled terminals in contact with the imaged spines. Spine motility was blocked by latrunculin, which prevents actin polymerization, and was disinhibited by blockade of action potential discharges with tetrodotoxin. It is proposed that an active presynaptic terminal restricts motility of dendritic spines.

Key words: dendritic spines; confocal microscope; EGFP; FM4-64; calcium; actin; latrunculin

The analysis of the rules that govern the development of dendritic spines may provide important clues concerning the role of dendritic spines in neuronal integration, which has been the focus of numerous studies in recent years. Major issues, including the role of presynaptic or postsynaptic activity in formation and stabilization of the synapse and the spine are still unsettled. Also, it is not entirely clear if spines are formed first as filopodia and search for a presynaptic partner, followed by formation of synapses (Jontes and Smith, 2000), or if spines are formed from shaft synapses by emerging after formation of a functional connection (Fiala et al., 1998). In that context, the conditions leading the transformation of a shaft synapse to a spine synapse are not entirely clear. Recently, dendritic spines have been shown to express a surprising amount of actin-dependent local motility not necessarily related to formation or growth of synapses, or their functioning (Fischer et al., 1998, 2000). This local motility has been found in different cell types (Dunaevsky et al., 1999) *in vitro* as well as *in vivo* (Lendvai et al. 2000). Still, it is not known what regulates this fine motility or how motility relates to synaptic function. We have analyzed age-dependent spine motility in dissociated cultured hippocampal neurons, attempted to correlate it with transient postsynaptic calcium variations, and with presence of a presynaptic terminal on the imaged spine. We conclude that spine motility is governed by presynaptic activity in the developing neuron.

MATERIALS AND METHODS

Tissue culture. Prenatal embryonic day 19 (E19) rat hippocampal neurons were dissociated and plated on polylysine-coated glass coverslips as described elsewhere (Papa et al., 1995; Murphy and Segal, 1996). In some experiments, neurons were cultured from the hippocampus of newborn transgenic mice, expressing enhanced green fluorescent protein (B5/EGFP; courtesy of J. Pickel, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD) (Hadjantonakis et al. 1998), as described earlier (Goldin et al. 2001). These cells were plated on glia bed and mixed at a ratio of 1:20 with nonfluorescent rat postnatal day 1 (P1) hippocampal neurons. Imaging experiments were conducted with 4- to 25-d-old cultures. A coverslip was transferred from the 24 well plate into the recording chamber, where it was perfused with recording medium containing (in mM): NaCl 129, KCl 4, MgCl₂ 1, CaCl₂ 2, glucose 4.2, and HEPES 10, pH was adjusted to 7.4 with NaOH, and osmolality to 320 mOsm with sucrose. The recording chamber was placed on the stage of an inverted confocal laser-scanning microscope (CLSM 510; Zeiss, Oberkochen, Germany). Individual pyramidal neurons were impaled with a micropipette containing Oregon Green (OG)-1 or calcein (Molecular Probes, Eugene OR; 10 mM stock solutions) that was iontophoresed into the cell. The pipette was withdrawn, and the dyes were allowed to equilibrate in the cell for 10–20 min. In the GFP cultures, spiny fluorescent cells were selected for observation, without their impalement with a micropipette. Individual dendritic spines were visualized at high power (Zeiss Plan-apocromat 100×, NA 1.4, or acroplan 63×, NA 0.9), and a subsection of the image, containing the region of interest of ~5 × 5 μm was imaged at a rate of 5–20 frames/sec. At the end of the experiment the culture was exposed for 45 sec to a medium containing 90 mM K⁺ (replacing equimolar amounts of Na⁺) and 10 μM FM4-64, and washed thereafter for 15 min with the standard recording medium, which did not contain Ca²⁺, to allow the dye to be trapped in the presynaptic terminals and not be released by spontaneous activity in the culture. The dendrite–spine segment was imaged again with two excitation wavelengths, to examine if a presynaptic terminal is attached to the imaged spine (Ziv and Smith, 1996). The presence of the FM dye in nerve terminals was verified by the observation that after replacement of the normal medium, trapped FM was released, and the label disappeared from the terminals within minutes. The two dyes were excited with two different wavelengths, 488 and 543, respectively, and care was taken to avoid “bleeding” of fluorescence from one channel to the other. The two lasers, argon and helium neon, respectively, were set at a low

Received Nov. 27, 2000; revised May 11, 2001; accepted May 18, 2001.

This work was supported by Grant 97/230 from the United States–Israel Binational Science Foundation and by Grant 1/76/691 from the Volkswagen Stiftung (Germany). We thank V. Greenberger for the production and maintenance of the cultures, Dr. J. Pickel (National Institutes of Health) for the GFP mice, and Dr. A. Bershadsky (The Weizmann Institute) for the gift of Latrunculin-B.

Correspondence should be addressed to Menahem Segal, Department of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel. E-mail: menahem.segal@weizmann.ac.il.

Copyright © 2001 Society for Neuroscience 0270-6474/01/216115-10\$15.00/0

Figure 1. Age-dependent motility in GFP-containing neurons. *A*, P1 cell grown in culture for 6 d expresses considerable spine motility. Two images on the left are successive, taken 5 sec apart, showing several dendrites, and spines in focus on one of them. On the right is a subtracted image, showing in black and white a disparity between the two left images, with gray showing the same position of the dendrite in the two images. Note that most of the spines stain black or white and that the parent dendrite is primarily gray. *B*, GFP-containing cell, grown for 2 weeks in culture, same series of images as in *A*. Much less motility is detected in the subtraction image on the right. *C*, Same conditions, 30-d-old culture, showing mature dendritic spines, expressing little or no distinct motility. Below, computer-generated contour frames taken from successive images of the spine shown in *C* with arrow, to illustrate little or no lateral motility of the spine. Scale bars: *A–C*, 3 μ m; *C*, bottom, 1 μ m.

intensity, 0.1–0.2% of nominal intensity, to avoid photodynamic damage or bleaching. For that reason, most of the experiments were conducted at a single image plane.

In some experiments, the culture was fixed with paraformaldehyde (4%) and immunostained for detection of synaptophysin. The previously studied, EGFP-labeled neuron was identified, and synaptophysin-containing puncta were imaged using Alexa 546 nm dye. The number of FM4-64 terminals was compared with the number of synaptophysin-containing terminals on the same dendritic branches.

Analysis. Spine motility was quantified by comparing the position of the spine in space over successive images. Spines could, in general, elongate, shrink, change diameter, or change position in space relative to the spine neck–dendrite. To simplify the quantification, the largest absolute disparity between two images, reflecting the change in position of the spine in space, was estimated by subtraction of one image from another. The maximum deflection in 100 sec of observation was measured for each spine and was expressed in micrometers per 100 sec. Zeiss-based Image software was used to standardize the quantification of shape changes. Likewise, the presence of calcium transients was quantified, counting significant deviations of fluorescence ($\Delta F/F$) in the spine and its parent dendrite. Although fluorescence levels were higher during a calcium event, that did not affect the estimation of spine motility, as seen below. Movements of all the spines of the same age group were averaged.

RESULTS

EGFP-labeled cells

EGFP-labeled neurons were imaged at 1, 2, and 5 weeks in culture. The youngest cells had a characteristic simple dendritic tree, with few secondary dendrites, on which there was a wide range of different dendritic appendages, from short dendritic spines, to very long, highly motile dendritic filopodia. A filopodium

occasionally ended with a growth cone-like structure at its tip, which exhibited long movements including extension and retraction over several minutes of observation. In the same neurons, dendritic spines, seen as short, 1–2 μ m in length, thick, occasionally with a distinct head, were also highly motile, expressing pivotal rotations, as well as rapid changes in the volume of their spine heads (Fig. 1*A*). These changes in position of the spine in space were evaluated by subtracting successive images from each other (Fig. 1*A3*). Further quantification was conducted with the individually labeled cells (below).

Most of the extensive movements subsided in the 2-week-old neurons. At this age group, there were fewer filopodia, and most of the spines expressed only subtle vibration-like fast variations in size or position in space. Little motility of any kind was detected in the 4- to 5-week-old cultures. Thus, even for the same size of spines, they were far more motile in the 1-week-old cells than in the older ones. The factors that control spine motility at the different age groups was further studied in cells injected individually with a fluorescent dye, as follows.

Age and motility

As seen with the GFP-labeled neurons, cells injected with OG-1 were highly motile in the young cultures compared with their older counterparts (Fig. 2*A*). The magnitude of movement decayed exponentially with time in culture (Fig. 3*A*). Because spines were also longer in the younger cultures, this age-dependent motility could be correlated with the initial size of the spines (Fig. 3*B*), thus, the longer the spine, the larger were its movements, and

Figure 2. Lack of correlation between spine motility, calcium variations, and afferent innervation. Cells were loaded individually with a calcium dye, OG-1. At the end of the observation time, cultures were exposed to the membrane dye FM4-64 to identify presynaptic terminals. **A**, Low-power view of a cell taken from a 2-week-old culture, showing in green the dendritic field of the cell, soma on the right edge, and in red, FM4-64 puncta. Scale bar, 10 μ m. **A1**, **A2**, High-power images of dendritic segments taken from **A**, indicated by arrows (**1**, **2**). Each image is a composite picture taken at two time points, at which the dendritic spines are at their farthest spatial positions within the 100 sec of observation. Red indicates one position, green indicates the other position, and yellow denotes overlap in the two positions. The main difference between **A1** and **A2** is in the presence of FM4-64 dots in **A2** and absence of dots in **A1**. **A3**, **A4**, Three-week-old culture, showing different images on top and the same images counterstained with FM4-64 at the bottom. A large FM particle is seen adjacent to the short spine on the left, and a small dot is seen adjacent to the long spine on the right. The difference image on top shows little movement of the left spine and a large movement of the right spine. **A4**, High-power image of a medium-sized spine, adjacent to an FM dot to show little disparity in position of the spine in space over time. Scale bars: **A1**–**A4**, 1 μ m. **B1**–**B4**, Correlating movement and calcium transients over time. Spines correspond to those shown in **A1**–**A4**, with the notations **S** for spine, **D** for dendrite, **S1** for spine 1, and **S2** for spine 2. Overlapped are the calcium transients, in continuous lines, quantified as $\Delta F/F$, and the movement is indicated with dotted lines. A clear lack of correlation between the calcium transients and local motility is seen. In general, calcium transients in the spine head and the parent dendrites are correlated, with notable exceptions. Larger variations in calcium transients are seen in **B2** compared with **B1**, as well as a large disparity between adjacent spines. In **B3**, a comparison between the mobile and the immobile spines indicates that although both can express simultaneous calcium transients, the long spine is more independent from the parent dendrite than the short one. **B4**, At a

high temporal resolution, large dendritic calcium events are always seen also in the spine, whereas the opposite is not always true, and independent calcium transients can be seen in the spine. Altogether, the spine expresses little motility compared with the other ones.

the relation to age by itself may be trivial. Still, even in the same cell (Figs. 1, 2*A1*), short spines could be as motile as long ones, indicating that size by itself does not determine spine motility.

Because spines could vary in motility even in a single neuron (Fig. 2, compare *A1*, *A2*), it is apparent that age by itself is not critical for spine motility. Two other factors can contribute to this, presynaptic and/or postsynaptic activity. Both were examined herein. To study the role of active presynaptic terminals, we stained the culture with FM4-64 and reanalyzed spine motility (Figs. 2, 4). In general, fewer FM-labeled terminals were associated with spines in the younger cultures than in the older ones.

Spine motility was inversely related to presence of FM puncta (Fig. 4). Both the magnitude of movement and the proportion of motile spines were markedly reduced by the presence of an active terminal (Fig. 4*A*, compare FM– and FM+). In that context, the length of spines was not correlated with presence of FM labeling (Fig. 4*B*).

For analysis, we grouped the spines into three ages, 5–8, 9–14, and 16–25 d *in vitro*. Only 31% of the young spines (26 measured) were associated with an FM puncta, and most of them (81%) were motile. Of the stationary spines, 80% had FM puncta attached. Most of the spines of the middle age group, (62% of 58 analyzed

Figure 3. Motility, length, and frequency of calcium transients as a function of age in culture. *A*, Maximal motility over 100 sec of observation, as a function of days in culture; $n = 293$ spines grouped in single days *in vitro*. *B*, Spines are longer in young cells than in older ones. The total population of spines was divided into two age groups, older and younger than 2 weeks *in vitro*. *C*, Frequency of calcium transients in 100 sec of observation time in the two age groups. $n = 157$ spines in the young group and 107 spines in the older group.

spines), were associated with a presynaptic terminal, and only a third of them (34%) were motile. Most of the stationary spines were associated with FM puncta (87%). A similar result was obtained with the older cells (58 spines analyzed, 74% touching FM puncta, 28% motile, most of the stationary ones were touched by FM puncta). These results indicate that under our testing conditions, spine movements are negatively correlated with the presence of an active presynaptic terminal.

Because FM staining varied in size even within the same field, we attempted to correlate the size of FM puncta with spine motility. There was a clear relationship between the two parameters, with the spine more likely to move if the FM puncta were small ($<0.3 \mu\text{m}$ in diameter) (Figs. 2*A3*, 5*A,C*). However, if the spine did move, there was no difference in the magnitude of the movement between small and large FM-associated spines (Fig.

5*B*), but in either case the extent of movement was one-third of that seen in the FM-negative spines.

FM and synaptophysin staining

In a previous study, Fischer et al. (1998) assert that motility is recorded also in spines that are contacted by a terminal immunostained retrospectively with an anti-synaptotagmin antibody, indicating that spines can be innervated by presynaptic terminals and still maintain motility. We therefore analyzed the relations between FM labeling and presence of presynaptic terminals stained with an anti-synaptophysin antibody. In a study of 473 spines (on 58 dendritic segments) (Fig. 6) we found that FM labeling comprises only a subset of synaptophysin-labeled terminals. Whereas $78.25 \pm 3.3\%$ of all the spines were contacted by synaptophysin-labeled terminals, only $54.9 \pm 3.7\%$ of these identified spines were also contacted by FM puncta. The fact that a substantial proportion of the terminals do not take up the dye is likely to indicate that they are not active. This may reflect the difference in motility scores between the two studies.

Calcium and motility

The role of postsynaptic activity in spine motility was examined in the different age groups. Calcium transients, resulting from either synaptic activity or backpropagating action potentials were recorded in nearly all the spines. Calcium transients were more frequent in the older cultures (10–25 d *in vitro*) than in the young ones (Fig. 3*C*). Altogether, calcium events were more numerous in spines touched by an FM puncta than in “naked” spines (Fig. 4*A*). Thus, calcium transients were more numerous in stationary spines than in motile ones. Still, for the same neurons (Fig. 2) in which backpropagating action potentials should in principle be the same at all branches, the innervated spines expressed more calcium transients than the non-innervated ones. This is likely to be attributable to the added synaptically evoked calcium events in the innervated (i.e., the FM-attached) spines. This can be estimated from the comparison of spine and dendritic-recorded calcium events. In the noninnervated spines all the calcium events are correlated with the dendritic events, whereas the innervated spines exhibited numerous events that were not paralleled by a calcium change in the dendrite (Fig. 2). Interestingly, spines expressing moderate amounts of calcium transients exerted the largest movements, whereas spines with few or many calcium transients were more stable (Fig. 4*A*). In none of these cases was there a direct correlation between presence of a calcium transient and spine movement.

Motility and actin

To examine the role of actin in spine motility, cultures were exposed, after initial imaging, to the actin polymerization blocker latrunculin (Allison et al., 1998). Within 3–5 min of application of the drug, spine motility ceased, whereas calcium events continued as before, both in the spine head and the dendritic shaft (Fig. 7). The effects of latrunculin were not washed out for the duration of the observation period, up to 30 min of observation time. Although gross spine motility stopped, the shape of the spine underwent a continuous shrinkage, to become shorter and more round (Fig. 7*A*).

The role of ambient temperature in spine motility was measured in EGFP-labeled cells. The same set of spines was imaged at 24, 30, and 36°C. In five cases studied (Fig. 8) spine motility was not affected much by ambient temperature. Once again, in the same cultures, spines that were not touched by FM puncta exhibited higher motility than the ones that were (Fig. 8).

Figure 4. Relationship between FM4-64 puncta, calcium transients, and motility. Calcium transients were divided into three categories: *low*, 0–1 events per 100 sec; *medium*, 2–6 events per 100 sec; and *high*, >6 calcium transients in 100 sec. *A*, Both in the absence of FM4-64 (*left column*) and in its presence (*right column*), the largest movements are exerted by spines with a moderate number of calcium transients, whereas higher and lower rates of calcium transients are associated with relatively smaller movements of the spines. Note, however, that the presence of FM particles was associated with a reduction in the magnitude of movements by a factor of 10. *B*, By comparison, the initial length of spine is not correlated with the presence or absence of FM particles, nor with the frequency of calcium transients. *n* = 62, 23, and 15 spines for the FM–, low, medium, and high calcium transients, respectively. *n* = 42, 28, and 28 spines for the FM+ group, for the low, medium, and high rates of calcium transients, respectively. *C*, Same as in *A*, but when only of the subpopulation of the adult spines is analyzed (>14 d *in vitro*).

Finally, although there is no correlation between spontaneous activity and spine motility on a continuous basis, it is possible that ongoing spontaneous network activity reduces spine motility. Cultures were therefore exposed to tetrodotoxin (TTX), and the same spines were monitored before and during exposure to the drug. In three experiments, TTX caused a significant enhancement of dendritic spine motility (Fig. 9), irrespective of the presence of FM puncta near the imaged spines. This indicates that the presence of FM puncta suppresses spine motility only if ongoing synaptic activity is maintained.

DISCUSSION

The present experiments were designed to analyze the factors that regulate dendritic spine motility in cultured hippocampal neurons and to relate them to formation of synaptic connections. The main parameters studied include age in culture, presence of presynaptic terminals, and spontaneous variations in $[Ca^{2+}]_i$, as imaged in living neurons with time lapse photography in a confocal laser-scanning microscope. As observed in previous studies (Papa et al., 1995; Ziv and Smith, 1996; Dunaevsky et al., 1999), dendritic spines are more motile in the

younger cells, when they are also less innervated by afferent fibers than the more mature ones. Likewise, spontaneous electrical activity, nearly absent in the very young cells, developed over 2–3 weeks in culture. In the present studies we extended these observations, to assert that the major parameter responsible for spontaneous motility of spines is not age by itself, but that the presence of an active presynaptic terminal, assessed by FM4-64 dye uptake, arrests spine motility.

For sake of simplicity, we lumped together minute, vibration-type movement, described recently by Fischer et al. (1998, 2000) and the slower, growth-related larger movements seen before (Ziv and Smith, 1996). Because both movement types are likely to be mediated by actin polymerization and both are blocked by the polymerization-inhibiting agent latrunculin, there is no clear mechanistic distinction between these movements, and thus they seem to constitute two extremes on the same motility axis. Still, the large, growth-related movement may be instrumental in searching for available presynaptic terminals, and in establishing connections, whereas the small vibratory movements may be relevant to the local regulation of spine–dendrite interactions, as predicted before

Figure 5. The size of the FM particle affects spine motility. The FM particles touching the spines were divided into small (<0.3 μm in diameter) and large (>0.3 μm in diameter). A marked difference was found between the two groups in the percentage of motile spine (*A*), with far fewer motile spines associated with the large FM particle than with the small one. The magnitude of the movement, however, was not correlated with the size of the particle (*B*). Altogether, when the spines were divided into motile and stable groups (*C*), the size of FM particles was larger in the stable spines than in the motile ones.

(Volfovsky et al., 1999) as well as regulation of trafficking of membrane components (Shi et al., 1999).

Variations in $[\text{Ca}^{2+}]_i$ are related to spine motility in a complex way. Whereas calcium events do not leave a lasting impact on spine morphology, long-term reduction in frequency of calcium transients, resulting from blockade of action potential discharge, cause the resumption of spine motility. This has been seen on a slower time scale (Papa and Segal, 1996; Collin et al., 1997), in which cells exposed to TTX begin to express filopodia, not seen normally in older cells in culture. The converse is also true, when dendritic spines, that normally express high level of motility are exposed to low concentration of AMPA, they cease to move

Figure 6. FM puncta constitute a subgroup of synaptophysin-labeled terminals. EGFP-containing neurons were imaged, followed by staining for FM4-64 (red puncta in *A*). At the end of the experiment, the same culture was fixed and stained for synaptophysin (Sf) (red puncta in *B*). As seen in the two images, all of the FM puncta have a corresponding Sf-labeled terminal, but some terminals do not stain for FM. Arrows show an example of spine with Sf, but without FM, and arrowheads show an example of a naked spine. Scale bar, 3 μm . *C*, Summary diagram of 473 spines counted on 58 20 μm dendritic segments. The difference between the number of Sf terminals and FM puncta is highly significant ($p < 0.01$).

Figure 7. Blockade of actin polymerization reduces spine motility but does not affect spontaneous calcium transients. *A*, Tracing of spine size by a thresholding procedure and overlapping spine sizes in the two extreme positions across 1 min of observation before (1), 1 min (2), and 20 min (3) after exposure of the cell to 2 μ M latrunculin. Note that the disparity between the two positions of the spine disappear at 20 min. Note also the change in spine shape after latrunculin. *B*, Spines cease to move, but continue to express calcium transients after exposure to latrunculin. Before (*B1*) and 15 min after (*B2*) onset of exposure of the culture to latrunculin. Same scheme as in Figure 2; continuous and dotted lines denote calcium transients in the spine and the parent dendrite, and broken line denotes measurement of maximal disparity in spatial location of the spine. Scale bar, 0.5 μ m.

(Fischer et al. 2000). This latter observation may provide a clue to the difference between the current observations and those of Fischer et al. (1998), who describe age-independent local motility of dendritic spines. Because cultures may vary greatly in the amount of spontaneous activity, it is possible that in their cultures activity level is rather low and TTX will not have an impact, but when $[Ca^{2+}]_i$ is raised by depolarizing the cell with AMPA, motility will cease. In our system, spontaneous activity level is high, spontaneous motility is low, but when activity is reduced, motility resumes. It is still hard to reconcile the results of Dunae-vsky et al. (1999) with the current results and those of Fischer et al. (2000), because they were unable to see an arrest in spine motility, even in the presence of high concentrations of glutamate. Others have also seen shrinkage of spines with glutamate

(Halpain et al., 1998). Once again, differences in preparation may account for this difference in results.

Arrest of motility is usually associated with shrinkage of the spines, as seen after treatment with latrunculin (Fig. 5). A rise of $[Ca^{2+}]_i$ also causes shrinkage of spines (Segal, 1995; Halpain et al., 1998). Thus, treatments that cause a rise of $[Ca^{2+}]_i$ will cause shrinkage of spines and arrest of their activity. This generaliza-tion is not entirely correct, because it has been shown that a small increase in $[Ca^{2+}]_i$ or electrical stimulation (Maletic-Savatic et al., 1999) can sometime cause elongation of spines, and produc-tion of novel ones (Korkotian and Segal, 1999), rather than their shrinkage. However, treatment with tetrodotoxin may in fact cause an increase in growth of certain spine type (Bravin et al., 1999). Thus, a bimodal relationship between activity, $[Ca^{2+}]_i$,

Figure 8. Lack of temperature effects on spine motility. Five-week-old GFP-labeled cell was imaged at room temperature (*A*), warmed to 30°C (*B*) and to 36°C (*C*), and imaged for 1–5 min in each of the temperatures. At the end of the imaging session, the cell was counterstained with FM4-64 to identify presynaptic terminals. The two *left columns* indicate the images that were taken 15 sec apart, and the image column on the *right* is a subtraction image of the *two left* ones. Only a minor movement is seen with the *top left* spine, whereas the other ones were stable. Interestingly, the *top left* spine is the only one not associated with a presynaptic terminal.

Figure 9. Blockade of spontaneous electrical activity enables spine motility. Three-week-old cell in culture, labeled with calcein was studied herein. A dendrite containing several spines was imaged (*A*), as before, in control (*B*), after exposure to TTX for 1 hr (*C*), and after wash (*D*). In each of these three cases, a subtraction of two images, 20 sec apart, is shown. No spontaneous motility is seen in the normal conditions, whereas blockade of spontaneous activity enabled this motility as in *B*. Scale bar, 3 μ m.

and spine expansion and contraction has been proposed (Segal et al. 2000) to account for these differences. The functional correlate of a change in spine dimensions with respect to calcium handling ability of the spine has been studied recently (Majewska et al. (2000).

The apparent lack of correlation between calcium transients and motility may reflect the fact that these movements are seen close to the limit of optical resolution. We (Korkotian and Segal, 2001) have recently found that backpropagating action potentials produce a small and rapid spine twitch that may

have an important impact on reactivity of the spine synapse to afferent stimulation. This twitch is also sensitive to latrunculin, indicating that actin filaments are involved in all types of spine motility.

The comparison between the two types of experiments conducted in the present study yields possibly interesting conclusions. The EGFP-labeled cells were taken from P1 mouse pups, whereas the OG-1-labeled cells were taken from E19 rat embryos. Both types of cells expressed an age-dependent motility of their spines, but the P1 EGFP cells matured faster and ceased to express motility already at 2 weeks in culture, at a time when the E19 cells were still active. This indicates that although both cell types were maintained *in vitro* for 2 weeks, the age of the cells at plating makes a distinct difference with respect to the maturation of the cells in culture.

The lack of motility seen here in association with maturation of the spine and the presence of an active presynaptic terminal contrasts with results of Fischer et al. (1998, 2000) who demonstrate that even innervated spines, assessed by the immunostaining with presynaptic markers, are motile. Our results do agree on the general rule that an increase in activity, produced in their studies by activation of glutamate receptors, causes arrest of spine motility. The converse is seen in our studies; blockade of activity with TTX initiates spine motility.

How reliable is a morphological marker of a presynaptic terminal to indicate that the synapse is active? In a previous study we (Vicario-Abejon et al., 1998) found that the presence of presynaptic markers does not necessarily mean that the synapse is active; spontaneous synaptic activity was absent under conditions in which the synaptic machinery was intact. In a more recent study (Collin et al. 2001) we found that FM1-43 labeling is a good predictor of synaptic activity. A similar observation was also reported recently by Renger et al. (2001), who found that synapsin-labeled puncta precede by several days the appearance of FM1-43 puncta. In the current studies, a disparity between synaptophysin immunoreactivity and FM labeling was seen, indicating that not all morphologically identified synapses are in fact active. This, it is likely that a spine may have a presynaptic marker and not be electrically active, hence, be motile.

Finally, the cultured neurons contain both spine-like and filopodia-like structures. In the mature neuron the definition of a spine is simple; it has a distinct morphological identity with a neck, a head, postsynaptic density, and an attached presynaptic terminal. In the developing neuron, there is no obvious definition of a spine. Would the presence of a presynaptic terminal constitute a prerequisite for the definition of a spine? Obviously not, because the spine was originally described by Ramon y Cajal (see English translation, 1995) before presynaptic terminals were known, and irrespective of them. Also, presynaptic terminals were reported to innervate even filopodia (Fiala et al., 1998), and some spines are known to exist even in the absence of presynaptic fibers (Bravin et al., 1999), and so the presence of a presynaptic terminal by itself cannot be used as a criterion for a "spine." Lack of motility by itself cannot define a spine because motility can increase or decrease in the same spine, depending on ambient electrical activity, seen here and elsewhere (Fischer et al., 1998, 2000). The situation may be simpler if one conducts retrospective immunocytochemistry and identifies a spine as one containing spine-specific or synapse-specific immunogenes, e.g., spinophyllin (Feng et al. 2000) or PSD-95 (Friedman et al. 2000), but these

may also mark immature spines. Thus, although a distinction between a spine and a filopodium is intuitive, more subtle variations among small protrusions that do not conform to a traditional definition of a spine may cause unjustified generalizations with respect to properties of spines, e.g., motility. Thus, although a formal definition of a spine at the light microscope resolution is called for, a tentative working definition of a spine as a short, up to 2–3 μm , relatively thick protrusion, which is relatively stable in space and does not exert long growth-related motility, is a good approximation of the ultimate spine and can be used for the study of properties of spines in general.

REFERENCES

- Allison DW, Gelfand VI, Spector I, Craig AM (1998) Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. *J Neurosci* 18:2423–2436.
- Bravin M, Morando L, Vercelli A, Rossi F, Strata P (1999) Control of spine formation by electrical activity in the adult rat cerebellum. *Proc Natl Acad Sci USA* 96:1704–1709.
- Collin C, Miyaguchi K, Segal M (1997) Dendritic spine density and LTP induction in cultured hippocampal slices. *J Neurophysiol* 77:1614–1623.
- Collin C, Vicario-Abejon C, Rubio ME, Wenthold RJ, McKay RDG, Segal M (2001) Neurotrophins act at pre-synaptic terminals to activate synapses among cultured hippocampal neurons. *Eur J Neurosci* 13:1273–1282.
- Dunaevsky A, Tashiro A, Majewska A, Mason C, Yuste R (1999) Developmental regulation of spine motility in the mammalian central nervous system. *Proc Natl Acad Sci USA* 96:13438–13443.
- Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, Allen PB, Ouimet CC, Greengard P (2000) Spinophilin regulates the formation and function of dendritic spines. *Proc Natl Acad Sci USA* 97:9287–9292.
- Fiala JC, Feinberg M, Popov V, Harris KM (1998) Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J Neurosci* 18:8900–8911.
- Fischer M, Kaech S, Knutti D, Matus A (1998) Rapid actin-based plasticity in dendritic spines. *Neuron* 20:847–854.
- Fischer M, Kaech S, Wagner U, Brinkhaus H, Matus A (2000) Glutamate receptors regulate actin-based plasticity in dendritic spines. *Nat Neurosci* 3:887–894.
- Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27:57–69.
- Goldin M, Segal M, Avignone E (2001) Functional plasticity triggers formation and pruning of dendritic spines in cultured hippocampal networks. *J Neurosci* 21:186–193.
- Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 76:79–90.
- Halpain S, Hipolito A, Saffer L (1998) Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *J Neurosci* 18:9835–9844.
- Jontes JD, Smith SJ (2000) Filopodia, spines, and the generation of synaptic diversity. *Neuron* 27:11–14.
- Korkotian E, Segal M (1999) Release of calcium from stores alters the morphology of dendritic spines in cultured hippocampal neurons. *Proc Natl Acad Sci USA* 96:12068–12072.
- Korkotian E, Segal M (2001) Spike-associated fast twitches of dendritic spines in cultured hippocampal neurons. *Neuron* 30:751–758.
- Lendvai B, Stern EA, Chen B, Svoboda K (2000) Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. *Nature* 404:876–881.
- Majewska A, Tashiro A, Yuste R (2000) Regulation of spine calcium compartmentalization by rapid spine motility. *J Neurosci* 20:8262–8268.
- Maletic-Savatic M, Malinow R, Svoboda K (1999) Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* 283:1923–1927.
- Murphy DD, Segal M (1996) Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. *J Neurosci* 16:4059–4068.
- Papa M, Segal M (1996) Morphological plasticity in dendritic spines of cultured hippocampal neurons. *Neuroscience* 71:1005–1011.

- Papa M, Bundman MC, Greenberger V, Segal M (1995) Morphological analysis of the development of dendritic spines in primary cultures of hippocampal neurons. *J Neurosci* 15:1–11.
- Ramon y Cajal S (1995) *Histology of the nervous system of man and vertebrates*, Vol 1. Oxford: Oxford UP.
- Renger JJ, Egles C, Liu G (2001) A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. *Neuron* 29:469–484.
- Segal M (1995) Morphological alterations in dendritic spines of rat hippocampal neurons exposed to NMDA. *Neurosci Lett* 193:73–75.
- Segal M, Korkotian E, Murphy DD (2000) Dendritic spine induction and pruning—common cellular mechanisms? *Trends Neurosci* 23:53–57.
- Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* 284:1811–1816.
- Vicario-Abejon C, Collin C, McKay RD, Segal M (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *J Neurosci* 18:7256–7271.
- Volfovsky N., Parnas H, Segal M, Korkotian E (1999) Geometry of dendritic spines affects calcium dynamics in hippocampal neurons: theory and experiments. *J Neurophysiol* 82:450–462.
- Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17:91–102.