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Rapid Actin-Based Plasticity in Dendritic Spines

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Summary

Dendritic spines have been proposed as primary sites of synaptic plasticity in the brain. Consistent with this hypothesis, spines contain high concentrations of actin, suggesting that they might be motile. To investigate this possibility, we made video recordings from hippocampal neurons expressing actin tagged with green fluorescent protein (GFP–actin). This reagent incorporates into actin-containing structures and allows the visualization of actin dynamics in living neurons. In mature neurons, recordings of GFP fluorescence revealed large actin-dependent changes in dendritic spine shape, similar to those inferred from previous studies using fixed tissues. Visible changes occured within seconds, suggesting that anatomical plasticity at synapses can be extremely rapid. As well as providing a molecular basis for structural plasticity, the presence of motile actin in dendritic spines implicates the postsynaptic element as a primary site of this phenomenon.

Introduction

Dendritic spines form the postsynaptic contact sites for the majority of excitatory synapses in the central nervous system (Gray, 1959; Peters et al., 1976; Parnavelas et al., 1977), making them ideally placed to act as the integrators of information flow in brain circuitry (Harris and Kater, 1994; Yuste and Denk, 1995; Shepherd, 1997). Based on their strategic location as bridges between axons and dendrites, spines have long attracted interest as potential mediators of the connective plasticity that is believed to underlie learning and memory (Eccles, 1979; Crick, 1982; Carlin and Siekevitz, 1983; Calverley and Jones, 1990; Lisman and Harris, 1993). This idea is supported by a substantial body of evidence indicating that changes in spine shape are correlated with alterations in behavior occurring in a variety of circumstances, ranging from learning paradigms to hormonal status (Ruiz Marcos and Valverde, 1969; Fifkova and Van Harreveld, 1977; Coss and Globus, 1978; Gould et al., 1990; Withers et al., 1993; Moser et al., 1994; Comery et al., 1996).

Despite widespread interest in this phenomenon, very little is known about the cellular mechanisms that regulate spine morphology. One reason for this is that mature spines are extremely small, being 1 μm or less across, making it difficult to resolve them by light microscopy, which is the only means of visualizing dynamic events in living neurons. To overcome this problem, we have taken advantage of the fact that dendritic spines are extremely rich in actin (Fifkova and Delay, 1982; Matus et al., 1982; Cohen et al., 1985) and that transfected actin accummulates at these same sites (Kaech et al., 1997); we show here that this also occurs for actin labeled with green fluorescent protein (GFP), allowing the direct visualization of spine dynamics. Using this approach, we report here the direct visualization of continual actin-driven shape changes in dendritic spines that are part of synaptic contacts in living hippocampal neurons. As well as providing a general mechanism for morphological plasticity in spines, the unexpected rapidity and extent of these changes have significant implications for synaptic plasticity in the brain.

Results

GFP-Labeled Actin Accurately Reveals Actin-Enriched Structures in Neurons

GFP fusion constructs were prepared for both the β- and γ-cytoplasmic actin isoforms, both of which are endogenously expressed in brain and are concentrated in dendritic spines (Kaech et al., 1997). Initially, both N- and C-terminally tagged constructs were tested by transfection in fibroblastic cells, where they labeled actin filaments whose motile behavior could be followed in video time-lapse recordings (Figure 1A; see supplemental videos at http://www.neuron.org/supplemental/20/5/847). GFP–actin was also transfected into neurons where after 2 days in culture fluorescent actin was concentrated in the growth cones of nascent axons and showed the same distribution previously described for actin in fixed neurons by staining with antibodies or rhodamine-phalloidin (Figure 1B) (Yamada et al., 1970; Forscher and Smith, 1988). Video recordings of GFP–actin fluorescence in growth cones mirrored the pattern previously described using phase or differential interference contrast microscopy, with thin fingers of filamentous actin extending into filopodia from their leading edges (Figure 1B; http://www.neuron.org/supplemental/20/5/847) (Bray and Chapman, 1985; Forscher and Smith, 1988).

As hippocampal neurons expressing GFP–actin matured over longer times in culture, their dendrites became studded with increasing numbers of fluorescent protuberances (Figure 2A), which at higher magnification showed morphologies typical of dendritic spines, including an actin-rich expanded head connected to the dendritic shaft through a narrow, more faintly fluorescent neck (Figure 2B). The appearance of these GFP–actin–labeled spines is the same as that previously visualized by staining spine-bearing neurons with antibodies against actin or rhodamine-labeled phalloidin (Matus et al., 1982; Kaech et al., 1997; Wyszynski et al., 1997). Figure 2C shows the confirmation of this identification in fixed cells counterstained with anti-actin. The appearance of GFP-labeled spines was not dectectably different from that of anti-actin–stained spines in control cells

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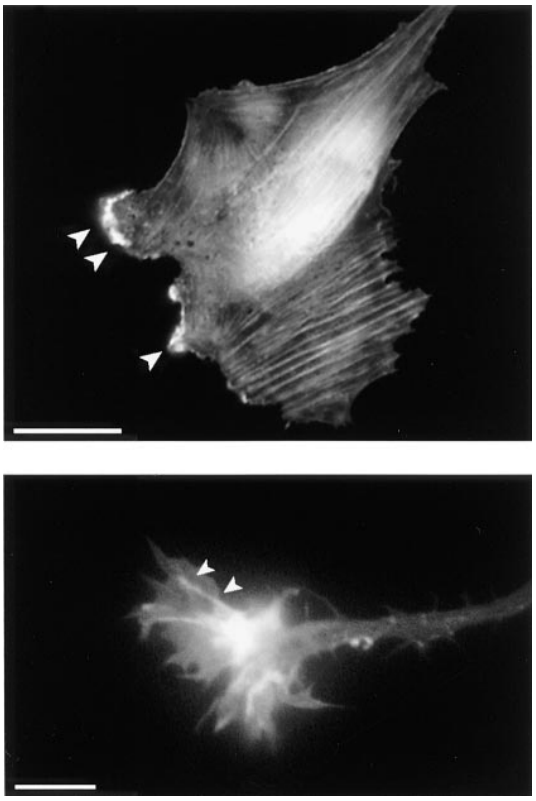

Figure 1. GFP-Tagged Actin Marks Actin-Containing Structures in Living Cells

(Top) A single frame taken from a video time-lapse recording (duration, 3 hr and 15 min) of a transfected rat embryo fibroblast cell expressing actin C-terminally labeled with green fluorescent protein (GFP). GFP–actin incorporated into stress fibers and structures associated with ruffling membranes at the cell's leading edge (arrowheads). This frame was taken 38 min from the beginning of the time-lapse sequence shown in the supplementary video data (see http://www.neuron.org/supplemental/20/5/847). Scale bar, 20 μm.

(Bottom) A single frame taken from a video time-lapse recording of a transfected hippocampal neuron expressing actin N-terminally labeled with GFP that had been in culture for 48 hr. Actin is more highly concentrated in the growth cone compared to the axon shaft and forms filamentous structures that penetrate into lamellipodia (arrowheads) and are seen to be dynamic in the supplementary video data. Scale bar, 5 μm.

from untransfected cultures (Figure 2D). Together with previous data for epitope-labeled actin (Kaech et al., 1997), this indicates that tagged actin effectively labels the actin in dendritic spines without altering their morphology.

Dendritic Spines Rapidly and Continuously Change Shape

Time-lapse recordings of GFP fluorescence in transfected neurons revealed that actin in dendritic spines is highly dynamic. The motility of actin in dendritic spines is an essentially dynamic property (see http://www.neuron.org/supplemental/20/5/847). In still images drawn from the recorded data, spine motility was represented by appreciable changes in spine shape that occurred between individual video frames taken several seconds apart (Figure 3A). Together with the original video data, this

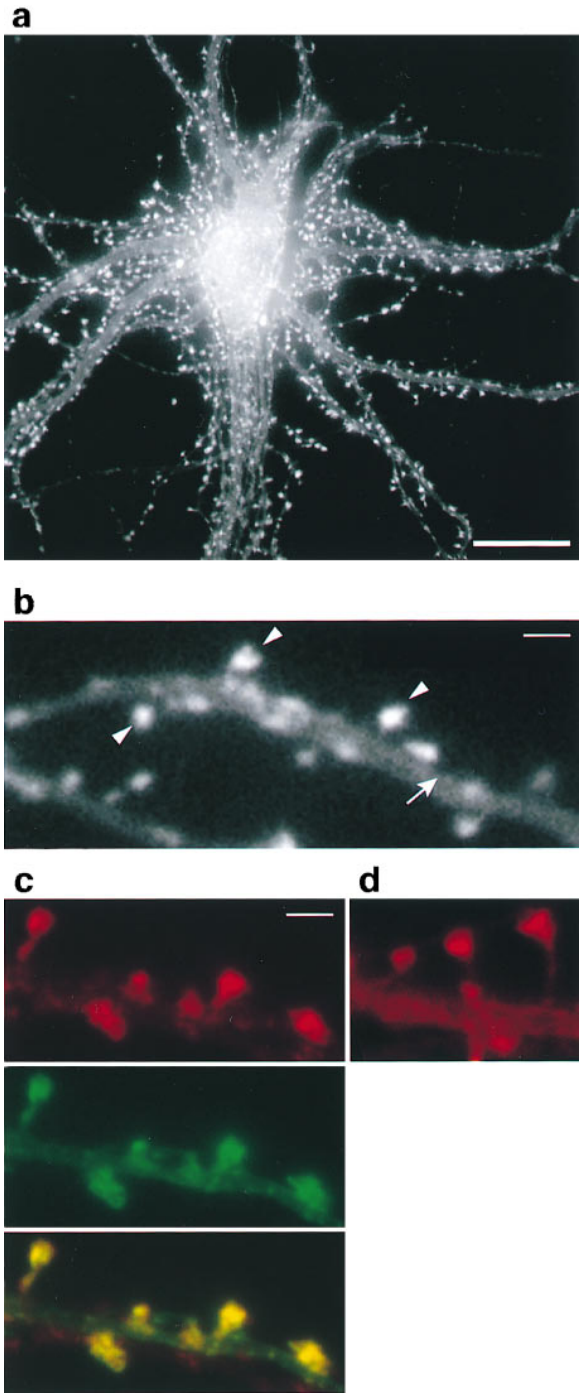

Figure 2. GFP–Actin Marks Dendritic Spines in Mature Cultured Neurons

(A) A single frame from a video time-lapse recording of a transfected cell expressing GFP–actin that had been in culture for 3 weeks. Dendritic spine heads appear as brightly stained points on the dendrites. Scale bar, 10 μm.

(B) Portion of a dendrite from a cell in another culture showing the high concentration of actin in spines (arrowheads) compared to the dendritic shaft (arrow). Changes in the shape of these spines during time-lapse recording are shown in the supplementary video data (http://www.neuron.org/supplemental/20/5/847). Scale bar, 2 μm.

(C and D) GFP–actin expressed by transfection marks actin in dendritic spines. GFP–actin–transfected (C) and untransfected (D) neurons were fixed and stained with antibodies against cytoplasmic

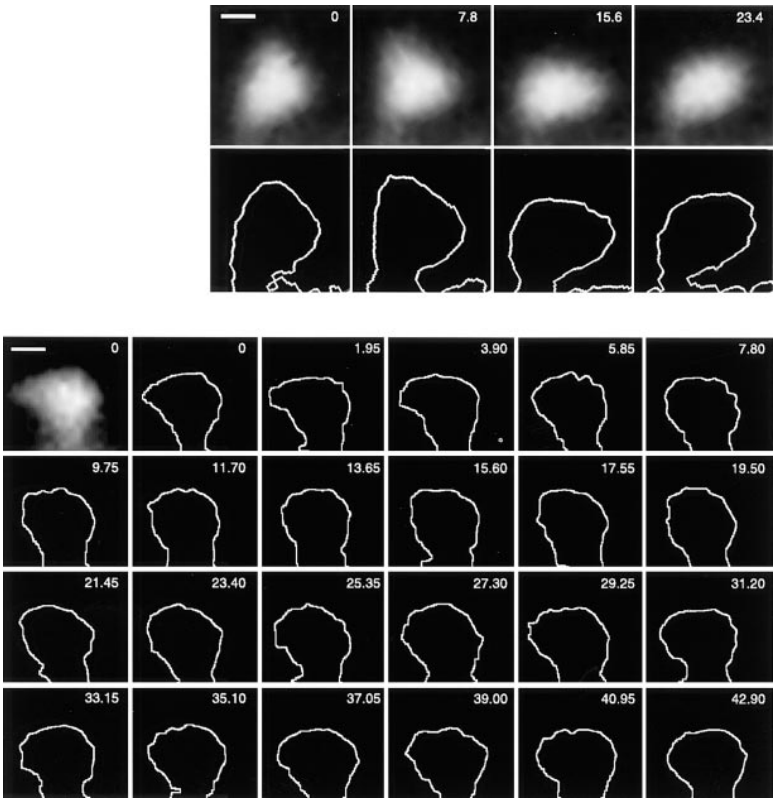

Figure 3. Rapid Variation of Spine Shape Revealed by Recording GFP–Actin Dynamics

(Top) The upper row shows four frames of GFP–actin fluorescence in spines of transfected hippocampal neurons, taken at the times indicated in seconds (top right). This data was taken from a video recording made as a continuous stream by integrating frames from the CCD camera for 1.5 s. The lower row shows spine profiles corresponding to the images in the upper row, derived by applying a thresholding routine to each frame.

(Bottom) Data from a second continuous stream recording of GFP–actin dynamics in spines. The original and profile images are shown for the first frame (top left), followed by profiles from successive frames taken at the times shown in seconds at the top right corner of each frame. Scale bar, 0.5 μm.

showed that spines changed shape constantly. To determine the extent of these morphological changes, we converted the original video data to outline profiles using an edge detection function. This is shown in the bottom row of Figure 3A, where four frames from a continuous recording of GFP–actin motility in a single spine are shown above their corresponding computed profiles.

Initially, we recorded dynamic events in spines by time-lapse video microscopy over periods of up to 30 min, demonstrating that actin-based spine motility is a robust and ongoing feature of dendrite function. Subsequently, we recorded at increasingly faster rates and ultimately found that substantial changes in the configuration of spine actin are detectable at even the fastest rate of recording possible, which used a 1.5 s integration time with no intervening delay. Changes in spine profile are more readily visible after the edge detection routine, as shown in Figure 3B where the first 43 s of a 2 min recording are shown.

Spine Dynamics Involve Changes in Shape, Not Size

Assessment of multiple video recordings suggested that the primary event in dendritic spine motility involves changes in spine shape rather than size. To confirm this, we collected fluorescence intensity data from individual spines simultaneously in two different ways: (1) within a box enclosing the entire spine and (2) within a smaller box placed across the border of the spine (Figure 4, top). Another large box was used to measure the background. Displayed graphically, the fluorescence intensities from these boxes provide a measure of total spine actin from the larger box and events involving changes in spine shape from the smaller edge box. The results show that although GFP–actin fluorescence intensity for the whole spine varied only slightly (open symbols), intensity within the smaller box (solid symbols) underwent large excursions that corresponded to the advance and retreat of the GFP–actin fluorescence signal at the spine boundary.

To assess the extent of these changes, we measured the dimensions of spine profiles in individual video frames in two orthogonal directions. The first measurement was taken in the direction of the spine neck, extended from the point where the spine joins the dendritic shaft to the edge of the head undergoing the largest excursion during recording, and the second measurement was made at right angles to this at the widest point of the spine head. The ratio between these two dimensions provides a criterion for overall spine shape, which was repeated for every frame in each series. The data in the top series shown in Figure 5 correspond to the profiles shown in Figure 3B, while the data below is taken from a second independent recording from another neuron. The results for six independently assessed spines are summarized in Table 1. They show that the average length and width of the spines during the recordings was greater than 1 μm but less than 2 μm. While the average excursion between frames over the duration of the recordings was between 2% and 4%, the

actin (top). Spine dimensions and overall appearance are not detectably different between transfected (left) and control (right) cells. The distribution of fluoresence from antibody-stained actin (top left) and transfected GFP–actin (center) overlap closely when displayed together (bottom). Scale bar, 2 μm.

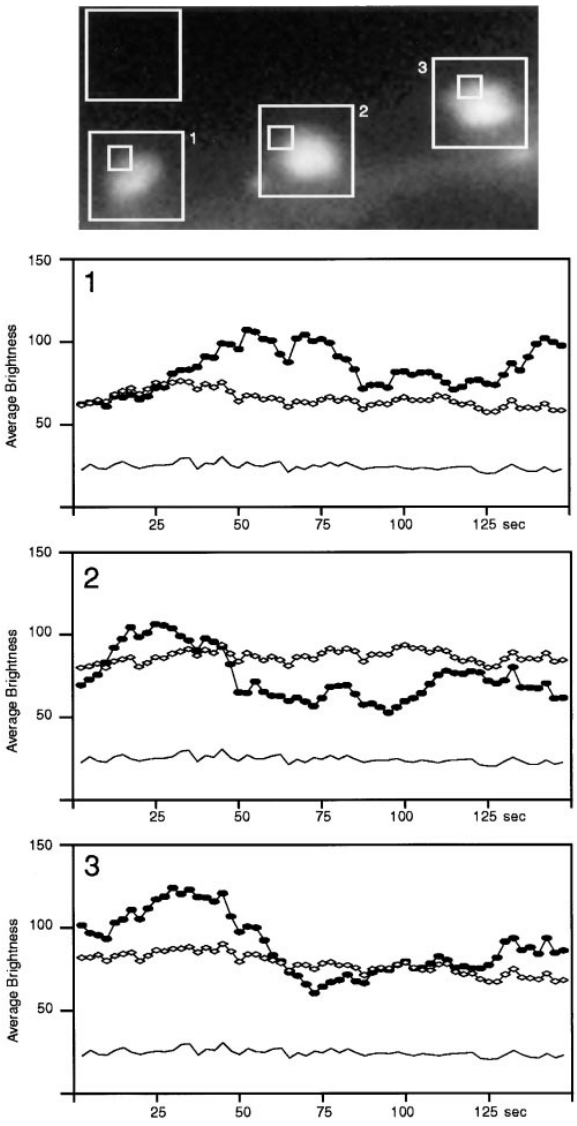

Figure 4. Changes in Fluorescence Intensity Are Associated with Alterations in Spine Shape

(Top) A single frame from a recording in which frames were taken every 1.5 s for 150 s shows three spines from which the data displayed in the graphs were derived. Fluorescence intensity was recorded from each spine either over the entire spine (large boxes) or from the edge of the spine (small boxes). Background fluorescence was recorded from a large box placed off the cell (top left). (Bottom) Graphical display of the variations in intensity over time for each of the three spines shown above during the 3 min of recording. The position of the boxes was constant throughout the recording period. Each graph is numbered to identify the corresponding spine in the upper panel from which the data was derived. Solid symbols, edge boxes; open symbols, whole spine boxes; line without symbols, background box. The original video recording for this figure is available as supplementary data (see http://www.neuron.org/supplemental/20/5/847).

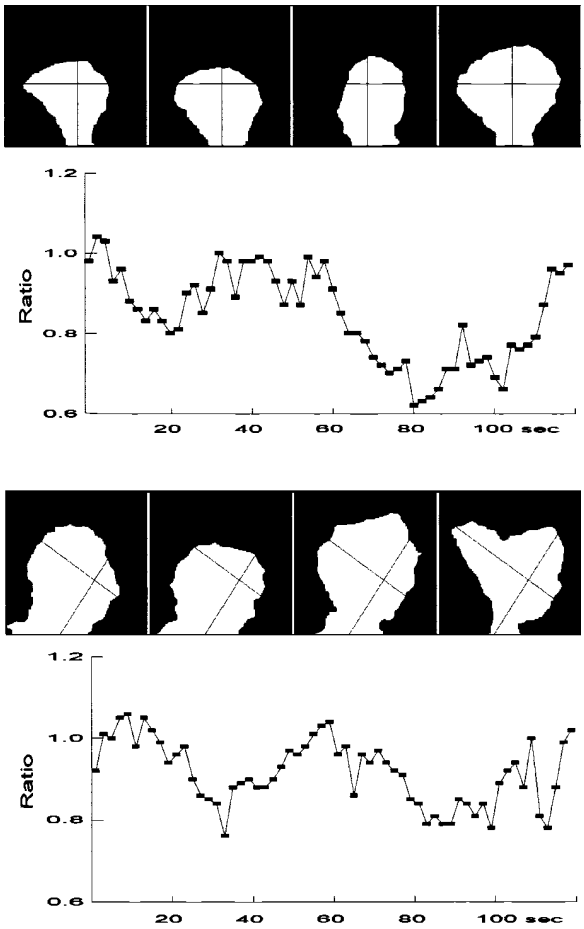

Figure 5. Assessment of the Extent of Changes in Spine Shape during Continuous Video Recording

Spine profiles were derived from continuous recordings of spine actin dynamics, and the length and width were assessed from lines superimposed on individual frames as displayed here for four frames from each of two independent recordings. The ratios of length to width for every frame in each recording are displayed in the two graphs.

maximal excursions were 3-fold higher. The differences between the smallest and largest measurements for spine length and width over the course of these 2 min recordings ranged from a minimum of 10% to a maximum of 31%. Thus, although spine dimensions are small, the changes that occur over even short intervals are surprisingly large.

Shape Change in Spines is an Actin-Dependent Process

To establish whether these changes in spine shape were actin-based, we exposed transfected cells to two drugs that interfere with actin polymerization, cytochalasin D and latrunculin B. Both compounds rapidly arrested spine motility (Figure 6; http://www.neuron.org/supplemental/20/5/847), and by titrating their concentrations, levels were found at which cytochalasin D reversibly blocked motility without significant depolymerization of spine actin (20 ng/ml). This can be seen at the top of Figure 6, where frames taken from recordings either during a control period without drug (left) or during drug treatment (right) show that there were no major changes in spine number or overall morphology.

To demonstrate these motility blocking effects in still images, we assessed changes in fluorescence intensity

Table 1. Data for Six Dendritic Spines

	Size		Maximal Excursion			
	Average		Between Frames		Overall	
Spine	Length	Width	Length	Width	Length	Width
1	1.0 μm	1.8 μm	6%	7%	26%	22%
2	1.7 μm	1.5 μm	8%	8%	19%	29%
3	1.2 μm	1.7 μm	8%	8%	18%	10%
4	1.1 μm	1.2 μm	18%	11%	39%	21%
5	1.3 μm	1.0 μm	9%	11%	28%	35%
6	1.1 μm	1.0 μm	10%	14%	20%	31%

Data for six dendritic spines, based on profile measurements as shown for the examples in Figure 5. The size columns show the average measurements for length and width over the entire series of sixty frames in each of the six recordings. The maximal excursion column shows the percent change in dimensions between frames or between the smallest and largest values over the entire recording of each spine (overall).

between consecutive frames of video recordings. This was done by subtracting pixel values from successive frames and averaging the differences to eliminate background noise. The results, for recordings made before and during drug treatment, are displayed at the bottom of Figure 6 as gray scale images. This procedure displays sites of motility in the video recordings as dark areas. Under control conditions, in the absence of drug, this produced multiple punctate sites along dendrites that corresponded to GFP–actin–labeled spines (seen at the top). The absence of such sites in the lower right image demonstrates that cytochalasin D inhibited spine profile changes.

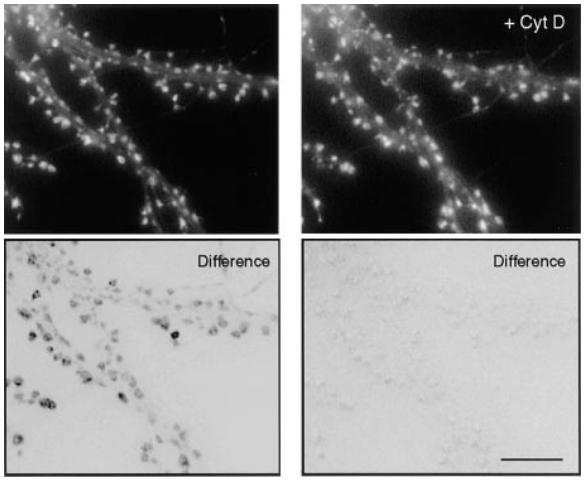

Figure 6. Drugs that Block Actin Polymerization Stop Spine Motility

The top row shows frames taken from a recording of labeled spines in a single transfected GFP–actin hippocampal neuron either before (left) or during (right) treatment with the actin polymerization–blocking drug cytochalasin D. The rapid motility evident in the control condition ceased during drug treatment without affecting overall spine morphology. This is most clearly demonstrated in the supplementary video data (see http://www.neuron.org/supplemental/20/5/847). To display it on the page, pixels in which changes in fluorescence intensity occured were averaged over 5 min of the control period (bottom left) or during drug treatment (bottom right) and displayed as gray scale images. Scale bar, 10 μm.

We also investigated the possible involvement of actomyosin-based contractile events in spine motility by treating cells with the myosin antagonists 2,3-butanedione monoxime (BDM) and KT5926. Unlike cytochalasin and latrunculin, these drugs were slow in onset and slowed, but did not completely stop, spine motility (data not shown). At the concentrations required for these effects, BDM and KT5926 are not specific to myosin, so it seems likely that their mode of action is indirect. We conclude that the actin-based motility in dendritic spines is driven mainly by polymerization.

Dynamic Spines Are Genuine Postsynaptic Structures

The transfected hippocampal neurons used to record actin dynamics in spines were cultured under conditions in which electron microscopy has shown that mature, innervated spines are formed (Bartlett and Banker, 1984). However, since immature dendrites make filopodia-like extensions (Papa et al., 1995; Dailey and Smith, 1996; Ziv and Smith, 1996), experiments were performed to demonstrate that the motile spines we observe are genuinely postsynaptic structures. To do this, we recorded spine dynamics in GFP–actin–expressing cells and then fixed them, while still on the microscope stage, and stained them with antibodies against several presynaptic vesicle marker proteins, including synaptophysin, synaptotagmin, and synaptobrevin, to confirm that they were contacted by axonal boutons. Figure 7A shows the GFP fluorescence image from a fixed cell in which the dynamic activity of the spines had been recorded prior to fixation. Figure 7B shows the corresponding image obtained by immunofluorescence staining with antibodies against synaptotagmin, and Figure 7C shows the combined image, revealing punctate synaptotagmin staining adjacent to GFP–actin–containing spines (arrowheads). As expected for a presynaptic marker, synaptotagmin is present in varicosities scattered along the dendrite but is absent from the dendrite itself (Figure 7B).

Discussion

Previous studies have indicated that dendritic spines contain high concentrations of actin (Fifkova and Delay, 1982; Matus et al., 1982; Cohen et al., 1985) and we have recently shown that the β- and γ-cytoplasmic actin isoforms, which are endogenously expressed by neurons, are specifically targeted to spines (Kaech et al., 1997). We took advantage of these circumstances by expressing GFP-labeled actin in cultured hippocampal neurons where, in agreement with our previous observations, it accumulated in spine heads, rendering them brightly fluorescent and enabling their dynamic activity to be captured by video microscopy. The results show that actin in spines is highly dynamic and, more unexpectedly, that the changes in spine shape driven by actin are large in relation to their size and and also rapid on an absolute time scale.

Changes in spine morphology were essentially limited

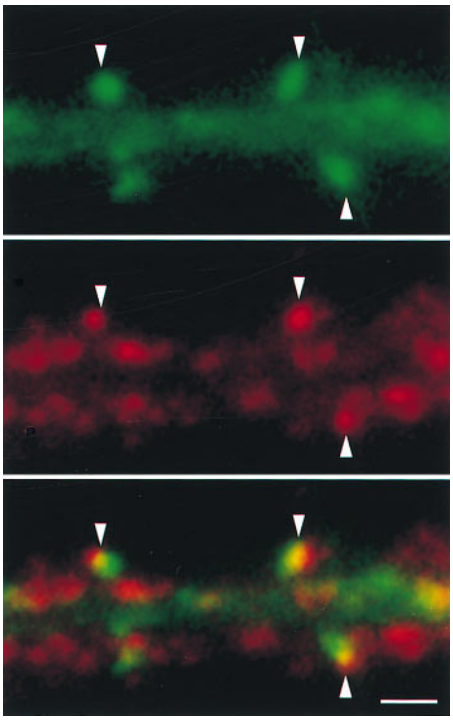

Figure 7. Motile Spines Are Contacted by Presynaptic Terminals

Fluorescence images of a dendrite segment from a GFP–actin–expressing hippocampal neuron with motile spines that was fixed in the observation chamber and counterstained with antibody against synaptotagmin to locate presynaptic specializations.

(Top) GFP fluorescence image showing bright GFP–actin–containing spines (arrowheads) on the more faintly fluorescent dendrite.

(Center) Anti-synaptotagmin staining of the same field showing varicosities (arrowheads) running along the surface of the unstained dendrite.

(Bottom) Combined image showing synaptotagmin-containing varicosities (red) apposed to GFP–actin–containing spines (green). The arrowheads are in register between the frames to indicate alignment. Scale bar, 2 μm.

to shape: over recording periods of up to 30 min, spines did not disappear or change noticeably in either volume or density. Alterations in spine size and density have been reported in both intact brain and organotypic brain slice cultures, but these typically occurred over a time scale of days (Hosokawa et al., 1992; Muller et al., 1993; Buchs and Muller, 1996), suggesting that changes in shape and those in size or number represent two distinct processes occupying different time scales. Although overall volume remained constant, spines manifested surprisingly large excursions in their linear dimensions, ranging from 10% to over 30% over a 2 min period of recording and being as large as 10% between successive images of single spines that were separated by only 1.5 s. Although they are small in absolute size, these changes are large when considered in relation to the dimensions of the synapse. Seen within the context of synaptic elements in the brain neuropil, a 10% change in spine dimensions could represent a substantial alteration in functional connectivity.

Spine motility also ceased rapidly when cultures were treated with drugs that block actin polymerization and recovered rapidly when these drugs were removed. This shows that the effects we observe are not the result of extraneous physical effects, such as thermal fluctuations, and at the same time identifies actin polymerization as the molecular mechanism responsible for changes in spine morphology. Further staining with antibodies against presynaptic marker proteins showed that motile spines are contacted by vesicle-containing axonal boutons, a result that is in agreement with previous studies showing that spines on hippocampal neurons cultured for several weeks are involved in mature synaptic contacts (Bartlett and Banker, 1984).

Given these now demonstrated rapid dynamics of spines, how might this affect synaptic efficacy? Two frequently discussed possibilities are that changes in spine shape may alter the influence of postsynaptic currents on the dendrite or that they affect second messengers, such as calcium, that are concentrated in spines (Lisman and Harris, 1993; Koch and Zador, 1993; Harris and Kater, 1994; Yuste and Tank, 1996). Further possibilities emerge from evidence that the state of actin polymerization influences the channel properties of NMDA-type glutamate receptors (Rosenmund and Westbrook, 1993). Ca^{2+} entering through NMDA receptor channels could initiate a feedback loop in which actin-driven changes in spine shape influence the efficacy of transmission at individual synapses. Calcium-sensitive interactions between the actin-binding protein α-actinin and subunit proteins of the NMDA receptor (Wyszynski et al., 1997) provide a further possibility for a regulatory mechanism connecting spine actin to postsynaptic function.

The surprisingly large extent of the shape variations we observe also warrants consideration in relation to anatomical plasticity at the synapse. While it has been widely assumed that alterations in neuronal connectivity must occur to account for the durability of memory, the growing axons and dendrites that would be necessary for major readjustments in circuitry have not been found in the adult nervous system. By contrast, changes in the shape, number, or density of dendritic spines following behavioral manipulations have been found in many studies involving a wide variety of animal species (e.g., Ruiz Marcos and Valverde, 1969; Fifkova and Van Harreveld, 1977; Coss and Globus, 1978; Calverley and Jones, 1990; Withers et al., 1993; Moser et al., 1994; Sunanda et al., 1995; Comery et al., 1996). In this respect, the most intriguing aspect of our results is the rapidity of shape changes in spines, with substantial alterations in the dimensions of individual spines being evident in the 1.5 s gap between successive recorded frames. This raises the possibility that the configuration of synaptic connections in the brain is in a constant state of actin-driven readjustment. It has been speculated that changes in spine morphology might bridge the gap between the electrophysiological events of sensory perception and the initial events of memory formation (Crick, 1982). Our results are consistent with this conclusion, and the possibility of visualizing cytoskeletal plasticity in dendritic spines using the techniques described here may now allow this hypothesis to be tested.

Experimental Procedures

Materials and Constructs

Cytochalasin D and 2,3-butanedione monoxime (BDM) were obtained from Sigma (St. Louis, MO); latrunculin B and KT5926 were

obtained from Calbiochem (San Diego, CA). Mouse monoclonal antibodies against synaptotagmin were kindly provided by Dr. T. Schaefer (Friedrich Miescher Institute, Basel), and purified IgG from rabbit polyclonal antibodies against cytoplasmic actins were kindly provided by Prof. U. Groeschel-Stewart (Technical University, Darmstadt, Federal Republic of Germany). Both were detected using appropriate Cy3-coupled secondary antibodies (Amersham, Zurich). GFP-tagged actins were prepared using plasmids carrying β- or γ-cytoplasmic actin cDNAs under the control of a β-cytoplasmic actin promoter (Kaech et al., 1997). The GFP coding region of pEGFP-N1 (Clontech, Palo Alto, CA) was fused in frame to the actin coding region either directly at the N terminus or at the C terminus after an 11 amino acid linker from vesicular stomatitis coat virus protein (Kaech et al., 1997).

Cell Culture, Transfection, and Microscopy

The rat embryo fibroblast cell line REF52 was grown under standard conditions in DMEM with 10% fetal calf serum. Cells were plated onto glass coverslips and transfected with plasmid DNA using Lipofectamine (Life Technologies, Basel). After the cells reached confluency, a scrape mark was made in the monolayer, and migrating cells were imaged at the edge of the wound over a period of several hours.

Neuronal cultures from E19 rat hippocampus were prepared and transfected as described (Kaech et al., 1995) and maintained in culture on glass coverslips for 3 weeks. For microscopy, they were mounted in purpose-built observation chambers (Type 1, Life Imaging Services, Olten, Switzerland) and imaged at 37°C on a Leica DM-IRBE inverted microscope using high numerical aperture oil-immersion lenses and a GFP-optimized filter set (Chroma Technology, Brattleboro, Vermont). Images were captured using a MicroMax cooled CCD camera (Princeton Instuments, Trenton, NJ) and MetaMorph Imaging Software (Universal Imaging Corporation, West Chester, PA). Except where otherwise stated, images were taken every 15 s with a 2 s exposure using neutral density filters to prevent photo damage. Drugs were applied in glia-conditioned medium by gravity-feed perfusion. For detection of presynaptic specializations, imaged cells were fixed directly in the observation chamber by perfusion with 4% paraformaldehyde in phosphate-buffered saline followed by indirect immunofluorescence staining with antibodies against synaptotagmin.

Image Analysis

Still images from individual frames of original video recordings were prepared using Metamorph 2.75 (Universal Imaging, West Chester, PA), and the panels were assembled with Adobe Photoshop. Spine profiles for Figure 3 were derived from thresholded images using the Sobel edge detection function in the Metamorph image analysis suite. Overall intensities of regions indicated by the white frames in Figure 4 were directly logged to Microsoft Excel for analysis. The length of spine heads was measured on thresholded images by laying the axis through the neck region as to reflect the largest change observed during the course of the recording, while the width scan axis was laid perpendicularly at the widest point of the spine head. To visualize the effect of cytochalasin, arithmetic difference images of 20 subsequent frames from recordings made prior to or during drug treament were averaged and inverted. Background levels of the resulting pictures were scaled to display the averaged differences in the original images as dark pixels.

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