

# Evidence for a Role of Dendritic Filopodia in Synaptogenesis and Spine Formation

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## Summary

Axo-dendritic synaptogenesis was examined in live hippocampal cell cultures using the fluorescent dyes DiO to label dendrites and FM 4–64 to label functional presynaptic boutons. As the first functional synaptic boutons appeared in these cultures, numerous filopodia (up to 10  $\mu\text{m}$  long) were observed to extend transiently (mean lifetime 9.5 min) from dendritic shafts. With progressively increasing numbers of boutons, there were coincident decreases in numbers of transient filopodia and increases in numbers of stable dendritic spines. Dendritic filopodia were observed to initiate physical contacts with nearby axons. This sometimes resulted in filopodial stabilization and formation of functional presynaptic boutons. These findings suggest that dendritic filopodia may actively initiate synaptogenic contacts with nearby (5–10  $\mu\text{m}$ ) axons and thereafter evolve into dendritic spines.

## Introduction

The initial establishment of physical contact between a particular axon and a particular dendrite is obviously a key step in axo-dendritic synaptogenesis. The cellular mechanisms that govern such synaptogenic contact, in turn, almost certainly play major roles in the developmental selection of synaptic partners and, thereby, in establishing the topology of functional neuronal networks. While it is well known that axonal growth cones guide elongating axons over long distances to their target areas, little is known about the short-range process by which prospective synaptic partners finally make their initial contact.

More than 90% of excitatory axo-dendritic synapses in the CNS occur on dendritic spines (Harris and Kater, 1994). Of these, many are formed between spines and varicosities along axons en passant. This morphological arrangement has suggested that one fundamental function of the dendritic spine is to bridge physical gaps between densely packed arrays of dendrites and en passant axons (Swindale, 1981). This arrangement also raises intriguing questions about the developmental origins of such axo-dendritic connections. For instance, might the spine or some dendritic precursor play a similar bridging role in the initial establishment of the contact? Unfortunately, in spite of the ubiquity and undoubted importance of spines in the mature nervous system, rather little is known about spine morphogenesis (see Harris and Kater, 1994). In particular, the timing of spine appearance in relation to the initial establishment of axo-dendritic contact remains unknown.

Chronologically, synaptogenesis and spine formation

are often preceded by a period during which numerous elongated filopodia extend from developing dendrites (Morest, 1969a, 1969b; Lund et al., 1977; Miller and Peters, 1981; Phelps et al., 1983; Ramoa et al., 1987; Ulfhake and Cullheim, 1988; Saito et al., 1992; Papa et al., 1995; Dailey and Smith, 1996). Structural similarities and the sequential appearance of dendritic filopodia and spines suggest that dendritic filopodia might be precursors of dendritic spines, but it is certainly not established that this is actually the case (see Purpura, 1975; Miller and Peters, 1981; Harris et al., 1989, 1992). Nonetheless, the elongated morphology of the dendritic filopodium suggests that it could serve as a bridging structure to facilitate axo-dendritic synaptic contact (Morest, 1969a, 1969b; Saito et al., 1992; Cooper and Smith, 1992; Dailey and Smith, 1996; see also Hinds and Hinds, 1972; Hayes and Roberts, 1973; Skoff and Hamburger, 1974; Vaughn et al., 1974; Pappas et al., 1975; Miller and Peters, 1981; Vaughn, 1989). Dendritic filopodia might increase the probability of chance encounters between an ingrowing axonal growth cone and a given dendrite simply by increasing the effective cross-section of that dendrite as a passive target. Alternatively, protruding dendritic filopodia could actively initiate contacts with nearby axonal shafts or growth cones.

The latter proposal, originally put forward in several earlier studies (Morest, 1969a, 1969b; Saito et al., 1992; Cooper and Smith, 1992), is supported by a recent confocal microscopy study of dendritic dynamics within a developing tissue environment. Dailey and Smith (1996) have shown that dendritic filopodia in hippocampal slices from neonatal rats exhibit a highly dynamic form of protrusive motility consistent with an active role in contact initiation. Dendritic filopodia were most abundant and dynamic during periods of rapid synaptogenesis, but both their numbers and motility declined thereafter. The decline in filopodial number was closely associated with an increase in the numbers of stable spine-like structures. Also, the apparent conversion of an actively protrusive dendritic filopodium to a more stable spine-like structure was occasionally observed. Unfortunately, their slice preparation did not permit directly correlated determinations of functional synapse formation.

To explore further the roles of dendritic filopodia in synaptogenesis and spine formation, we developed a dissociated cell culture system in which the location and function of individual synaptic boutons, as well as dendritic structure and dynamics, could be visualized in live rat hippocampal neurons. Using this system, we measured in vitro developmental time courses of both dendritic filopodial dynamics and synaptogenesis and tested for correlations between these two variables. We also succeeded in directly visualizing the formation of contacts between dendritic filopodia and nearby axons. Our results support the ideas that dendritic filopodia play an active role in the initiation of synaptic contacts and that they are direct precursors to dendritic spines.

## Results

The current study was guided by the hypothesis that dendritic filopodia actively initiate synaptogenic contacts (i.e., contacts that culminate in the formation of a synapse) and thereafter evolve into dendritic spines. This hypothesis suggests several testable predictions: first, dendritic filopodia should exhibit protrusive motility patterns consistent with a role in short-range exploration and contact initiation; second, dendritic filopodia should appear in numbers at least equal to the number of spines formed eventually on dendrites; third, the developmental disappearance of dendritic filopodia should coincide with the appearance of dendritic spines; fourth, the appearance of dendritic spines should coincide with the appearance of functional synapses; and fifth, synapses should be formed at contact sites between dendritic filopodia and nearby axons.

The predictions listed above were tested in primary cultures of dissociated rat hippocampal neurons. The relative simplicity of this preparation and its accessibility to experimental manipulation make it an attractive system for the study of synaptogenesis in the mammalian CNS (Bartlett and Banker, 1984; Craig et al., 1993, 1994; Fletcher et al., 1994; Basarsky et al., 1994; Verderio et al., 1994, 1995; Matteoli et al., 1995). Furthermore, since these cultured neurons form dendritic spines that closely resemble their counterparts in vivo (Papa et al., 1995), they should provide useful information about spine development. However, even in this relatively simple preparation, the details of dendritic structure are usually obscured by the complex mesh of neuronal processes and glial cells that forms in the culture dish.

To resolve fine details of dendritic morphology in hippocampal cultures, we developed a method to label single neurons with the fluorescent lipophilic dye FAST DiO. A micropipette was used to apply the dye to selected neuronal somata. Dye incorporated into the soma membrane rapidly diffused into the dendritic and axonal membranes, labeling entire neuronal surfaces within 20–30 min (Figure 1A). Dendrites were identified on basis of their distinctive morphology (Dotti et al., 1988) and by immunostaining (see below).

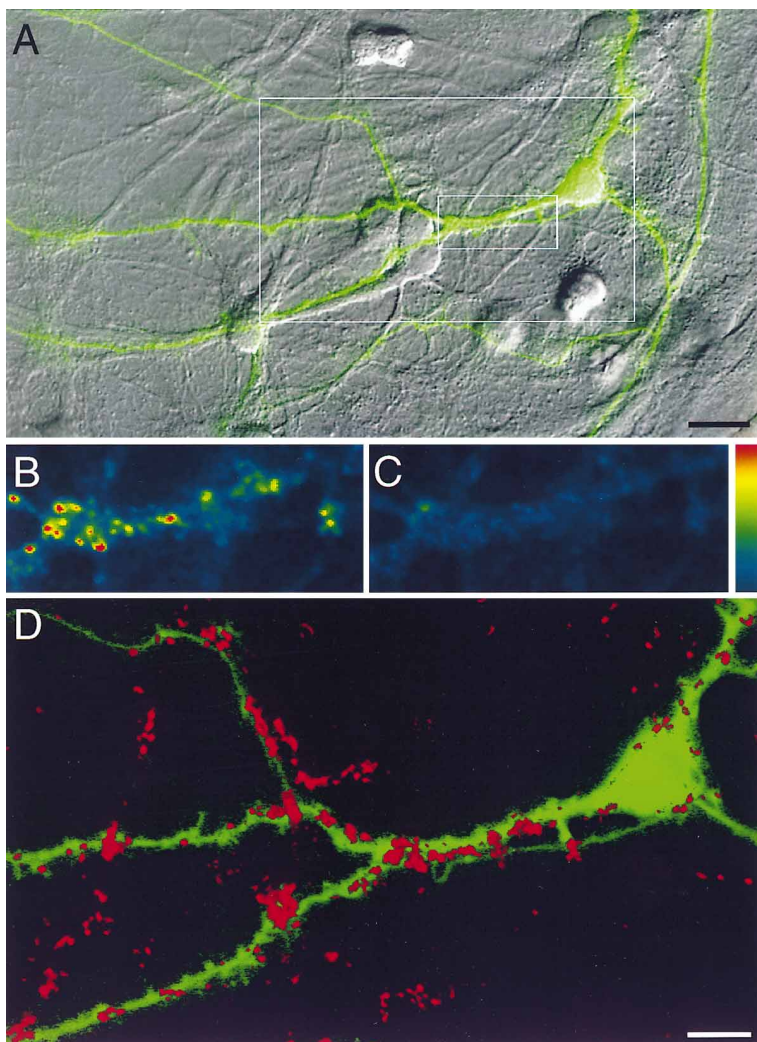
To visualize functional presynaptic boutons, we utilized the styryl dye FM 4–64, a fluorescent endocytotic marker that provides a quantitative measure of synaptic function (Henkel and Betz, 1995; see also Betz and Bewick, 1992, 1993; Ryan et al., 1993, 1995). Neurons were stimulated to fire action potentials in the presence of a solution containing 15  $\mu$ M FM 4–64. After the neurons were washed with dye-free solution, discrete fluorescent puncta, corresponding to presynaptic boutons, were clearly visible (Figure 1B). To correct for nonspecific dye uptake and dye binding, a second image was collected after an additional train of action potentials was used to release dye trapped in synaptic vesicles (Figure 1C). This image was subtracted from the first image, and the resulting difference image was overlaid onto the fluorescence image of the FAST DiO-labeled neuron (Figure 1D; see Experimental Procedures). These methods enabled us to follow time-dependent changes in dendritic structures and relate them to the occurrence and position of functional presynaptic boutons.

Since dendrites of only one cell were labeled in every locale, we were concerned that presynaptic boutons might be assigned to inappropriate postsynaptic partners, as other, unlabeled, dendrites in the field might be erroneously ignored. This would be particularly true for fasciculated dendrites but also for dendrite intersection points. Although we used differential interference contrast (DIC) images to select neurons with relatively few intersecting dendrites, the possibility that unlabeled dendrites in the field would remain undetected could not be excluded. We therefore performed retrospective immunostaining of the dendrite-specific microtubule-associated protein MAP-2 (Caceres et al., 1984) in order to compare the underlying dendritic network with that observed in DIC images of the same field. Figure 2A is a DIC image of a relatively complex field in a 45 days in vitro (DIV) preparation, and Figure 2B shows the digitally combined images of DiO, FM 4–64, and anti MAP-2 labeling of the same field. By comparing image pairs such as those shown in Figure 2 ( $n = 10$ ), we concluded that most of the MAP-2 stained processes could be detected in the DIC images as well. Furthermore, dendrites in these preparations did not appear to display a strong tendency to fasciculate. We therefore concluded that satisfactory determinations of synaptic association between dendritic structures and presynaptic boutons could be obtained from the combined use of composite DiO/FM 4–64 fluorescent images and DIC images.

### Dendritic Filopodia Exhibit Protrusive Motility Patterns Consistent with a Role in Short-Range Exploration and Contact Initiation

The dendritic filopodia of pyramidal neurons in developing hippocampal slices were shown previously to be highly motile protrusive structures that extend and retract repeatedly (Dailey and Smith, 1996). To determine if dendritic filopodia in hippocampal cell cultures display similar motility patterns, we performed time-lapse recordings of dendritic filopodia extending from the dendrites of young (9–11 DIV) neurons. We also compared their behavior with that displayed by dendritic protrusions in older (20–27 DIV) neurons. Our time-lapse observations revealed that the dendritic filopodia of young hippocampal neurons in culture were short-lived, extremely dynamic structures that repeatedly extend and retract, whereas dendritic protrusions of older neurons were persistent and relatively stable structures, as previously shown for older hippocampal neurons in brain slice preparations (Hosakawa et al., 1992, 1995; Dailey and Smith, 1996) and in culture (Segal, 1995).

These differences between dendritic protrusions of younger and older neurons are illustrated in Figure 3. This figure shows two time-lapse sequences at 6 min intervals of dendritic segments of an 11 DIV neuron and a 20 DIV neuron. Examination of these time-lapse sequences showed that most of the protrusions on the younger dendrite were transient structures, which extended rapidly from various positions along the dendritic shaft only to retract soon afterwards (Figure 3, left panels). In contrast, the dendritic protrusions of the older neurons were persistent structures, which showed little change over time (Figure 3, right panels).



**Figure 1.** Imaging of Dendritic Structure and Presynaptic Boutons in Live Cultured Hippocampal Neurons

(A) A fluorescence image of a single pyramidal neuron labeled with FAST DiO, digitally overlaid on a DIC image of the same field. The neurons shown in this figure were grown for 13 DIV prior to the experiment.

(B) A pseudocolor fluorescence image of presynaptic boutons loaded with FM 4-64. The area shown corresponds to the inner rectangle in (A). Fluorescence intensity is coded according to color bar on far right.

(C) The same field shown in (B) after the dye was unloaded by stimulating the neurons to fire action potentials for 60 s at 10 Hz.

(D) Digital superposition of the FM 4-64 difference image (red), created by subtracting the image in (C) from that in (B), onto the fluorescence image of the FAST DiO-labeled neuron (green). Area shown corresponds to outer rectangle in (A). Scale bars, 20  $\mu\text{m}$  (A) and 10  $\mu\text{m}$  (D).

We shall refer to the relatively stable protrusions seen on older neurons as spines, based on their morphological resemblance to stubby, thin, and mushroom-shaped spines previously described in the literature (see Papa et al., 1995) and the fact that many of them are directly associated with a presynaptic bouton (Figure 3B). Conversely, highly motile protrusions not associated with presynaptic boutons will be referred to as filopodia.

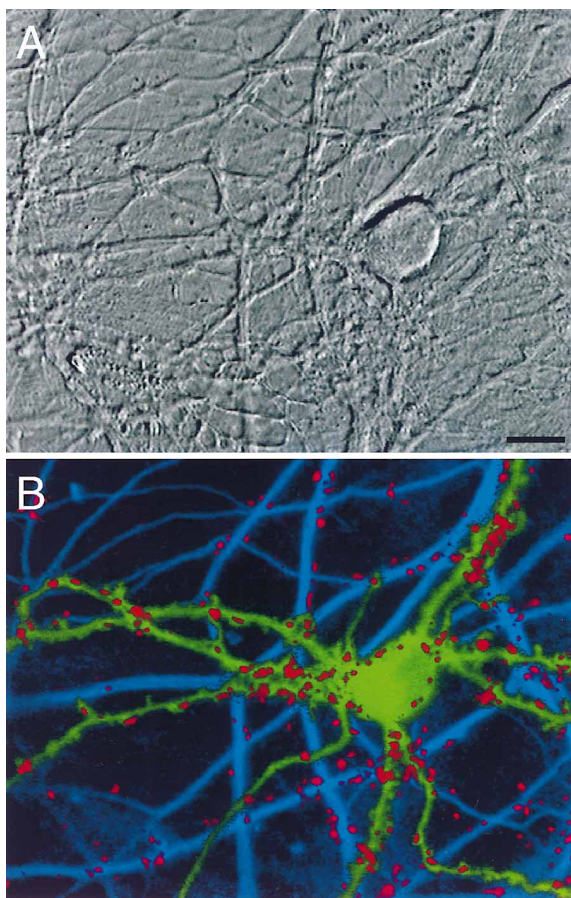
Filopodial protrusive activity defines a zone extending 5–10  $\mu\text{m}$  from the dendritic shaft that is periodically occupied by the filopodia of that dendrite. This is exemplified in Figure 3C. This figure was created by plotting, for each filopodium, a line proportional to the peak length it had attained during the time-lapse sequence in Figure 3A and suggests that dendritic filopodia are capable of exploring a considerable volume on all sides of a dendritic shaft.

Figure 4A portrays changes over time in the lengths of eight filopodia and eight spines from the dendritic segments shown in Figure 3. As shown in this figure, the lengths of dendritic filopodia extending from immature dendrites changed considerably over time, in one case transiently exceeding 10  $\mu\text{m}$  (Figure 4A, left panel). In

contrast, the length of dendritic spines on mature dendrites did not change much over time (Figure 4A, right panel).

The motility of a dendritic filopodium or spine was scored by averaging the absolute value of changes in length between sequential images over the entire time-lapse session (see Experimental Procedures). The ensemble motility data for 157 spines and filopodia followed in nine separate experiments is summarized in Figure 4B. This figure clearly shows that filopodia on immature dendrites of cultured hippocampal neurons are highly motile, while spines on mature dendrites are much more stable.

Transient dendritic filopodia on immature dendrites (9–11 DIV) had a mean lifetime of  $9.5 \pm 7.7$  min (mean and standard deviation, 63 filopodia, five experiments, same neurons as in Figure 4B). For this analysis, we necessarily considered only filopodia that both appeared and disappeared during the time-lapse session, while filopodia that were discernible either at the beginning or at the end of the time-lapse session (about 33%) were excluded. This selection procedure may discriminate against the more persistent filopodia, and our mean



**Figure 2.** Detection of Unlabeled Dendrites in the Vicinity of Labeled Dendrites

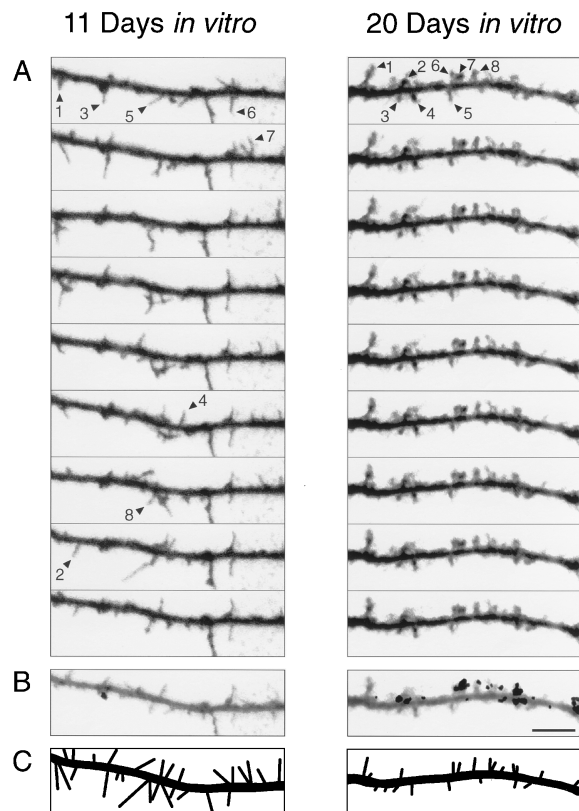
A single neuron in a relatively complex environment was labeled with FAST DiO, and presynaptic boutons were labeled with FM 4-64. The neurons were then fixed and immunostained with an antibody against MAP-2.

(A) A DIC image obtained prior to fixation.

(B) A digital composite of three fluorescent images of the same field showing the DiO-labeled neuron in green, the FM 4-64-labeled presynaptic boutons in red, and MAP-2 immunostaining in blue. Comparing the DIC and fluorescence images reveals that most of the MAP-2 stained dendrites are also discernible in the DIC image of the same field. Scale bar, 10  $\mu$ m.

lifetime values may therefore underestimate those that would be obtained with longer sampling periods.

Although spines on mature dendrites showed little change over time, we did observe, on several occasions, the abrupt extension of thin and transient filopodia from the distal tips of otherwise stable spines, including spines on which synapses had formed (data not shown; see also Dailey and Smith, 1996). We also observed the occasional extension of transient filopodia directly from the shaft of mature dendrites. It is important to note, though, that spines associated with presynaptic boutons were almost always persistent structures, displaying little change over time. Even in cases in which transient filopodia protruded from the distal tips of such spines, the original spine segments remained stable.



**Figure 3.** Time-Lapse Sequences of Dendritic Filopodia and Dendritic Spines

Two excerpts from time-lapse sequences of FAST DiO-labeled dendrites of 11 DIV (left panels) and 20 DIV neurons (right panels). The gray scale of these images was inverted to increase detail clarity. (A) Time-lapse sequences composed of 9 frames at 6 min intervals. Note the rapid changes in filopodium morphology compared with the stability displayed by dendritic spines (see also Figure 4A).

(B) Location of presynaptic boutons. A single frame from each time-lapse sequence onto which the corresponding FM 4-64 difference image, created at the end of the time-lapse session, was digitally overlaid. Presynaptic boutons are shown as dark black spots.

(C) Cumulative representation of all spines or filopodia in the 9 frames shown in (A). Each filopodium or spine is represented by a line proportional to the peak length it had attained during the time-lapse sequence. Note that the cumulative filopodium density of the 11 DIV neuron is greater than the instantaneous filopodium density observed in any single frame in (A). Scale bar, 10  $\mu$ m.

### Dendritic Filopodia Are Formed in Sufficient Numbers to Account for the Entire Spine Population

If dendritic filopodia are the developmental precursors of dendritic spines, the cumulative numbers of filopodia formed during development should match or exceed the numbers of spines observed on mature dendrites. Previous studies have reported that the density of dendritic filopodia is much lower than the density of mature spines (Morest, 1969a, 1969b; Papa et al., 1995). However, since filopodia are evidently short-lived and turn over at high rates, studies done in fixed tissue could have greatly underestimated the actual number of filopodia extended over the period of developmental synapse formation.



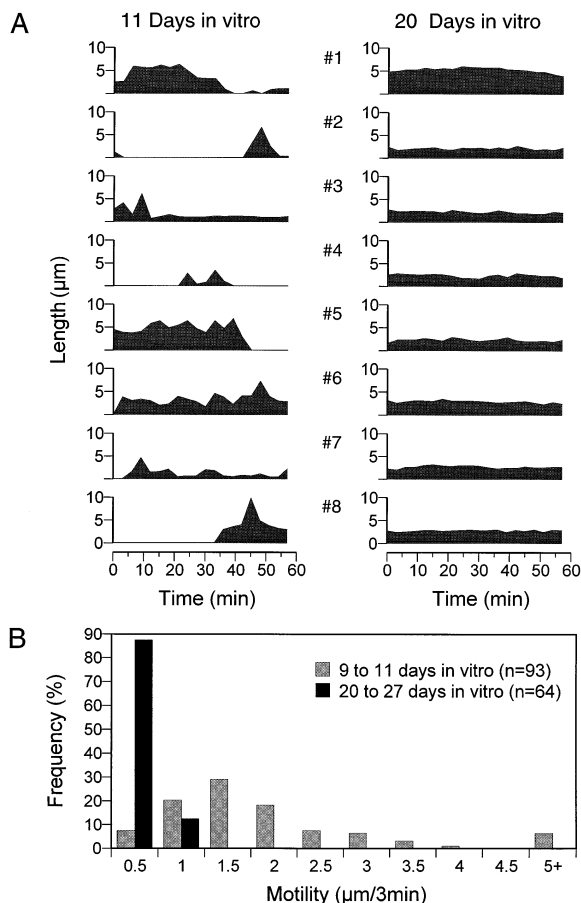


Figure 4. Dendritic Motility in Mature and Immature Neurons

(A) Changes in filopodium or spine length over time. The lengths of the numbered filopodia and spines seen in Figure 3 were plotted as a function of time from the beginning of the time-lapse session (20 timepoints, 3 min intervals). Left panels: filopodia of an 11 DIV neuron (corresponds to left panels in Figure 3). Right panels: spines of a 20 DIV neuron (corresponds to right panels in Figure 3). The spine lengths in the older neuron remained relatively constant during the time-lapse session. In contrast, the lengths of dendritic filopodia in the younger neuron changed rapidly over the same period.

(B) Distributions of dendritic protrusion motility in immature and mature neurons. The motility of a dendritic filopodium or spine was scored by averaging the absolute value of changes in length between sequential images over the entire time-lapse session (see Experimental Procedures for details). Normalized distribution for immature neurons (9–11 DIV, 93 filopodia) is shown in gray. Normalized distribution for mature neurons (20–27 DIV, 64 filopodia) is shown in black.

Graphic comparisons of filopodium and spine densities in young and old neurons are shown in Figure 3. Based on any single timepoint in Figure 3A, one might conclude that there are many more spines on the dendrite of the older neuron than filopodia on the younger dendrite. However, when this comparison is based on images representing the cumulative appearance of transient filopodia during an extended period, even as short as a period of 1 hr, the filopodium density easily exceeds the spine density in the older neuron (see Figure 3C).

To estimate cumulative numbers of filopodia on immature dendrites for comparison to numbers of spines ob-

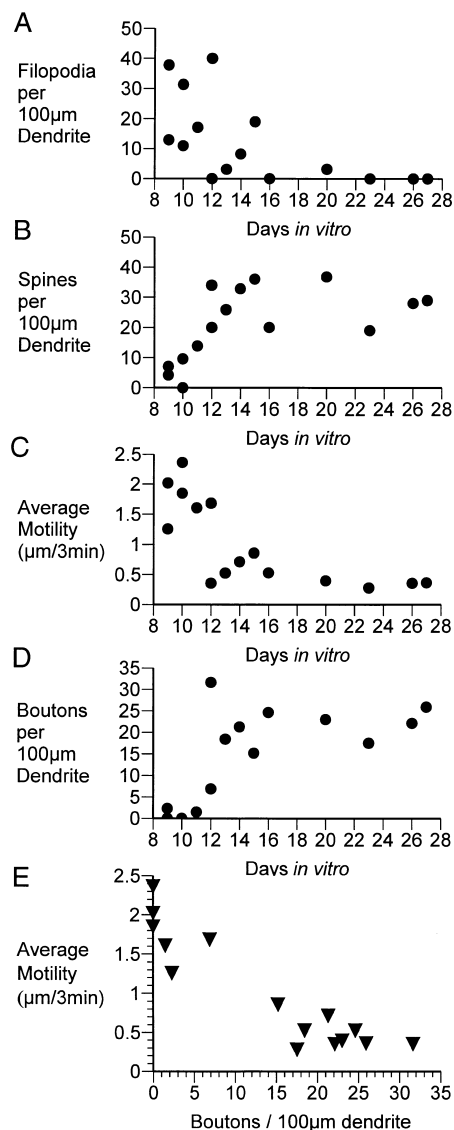
served at later stages, we observed the formation of dendritic filopodia over 1 hr time-lapse sessions. Cumulative filopodial densities were determined by counting all filopodia (no matter how short-lived) observed during the entire time-lapse session and dividing by dendritic segment length. Spine densities were determined in similar fashion, although cumulative and instantaneous densities were essentially identical here, owing to the inherent persistence of spines. This analysis was carried out on the dendrites of 10 DiO-labeled neurons in two groups (9–12 and 20–27 DIV). We found that the cumulative densities of dendritic filopodia in the younger neurons were quite comparable to the spine densities determined for the older neurons ( $38.8 \pm 15$  filopodia/100  $\mu\text{m}$  dendrite, 9–12 DIV;  $38.0 \pm 8$  spines/100  $\mu\text{m}$  dendrite, 20–27 DIV). Our 1 hr observation periods were quite short, however, in comparison to the duration of developmental synaptogenesis, so it seems safe to conclude that dendritic filopodia are formed along immature dendrites in numbers easily exceeding the numbers of dendritic spines ultimately formed in cultured hippocampal neurons.

#### The Disappearance of Filopodia Coincides with Appearance of Persistent Spines

To examine the suggestion that dendritic filopodia are the direct precursors of dendritic spines, we determined how the numbers of motile dendritic filopodia and persistent spines change with time in vitro. Dendrites of 15 neurons were observed by time-lapse microscopy for 1 hr. The resulting 1 hr cumulative filopodial densities were then plotted as a function of time in vitro (Figure 5A) and compared with the densities of stable spines on the same neurons (Figure 5B). For this purpose, stable spines were defined as protrusions with a motility score of 1  $\mu\text{m}/3$  min or less, while protrusions with higher motility scores were considered to be filopodia (see Figure 4B). A comparison of Figures 5A and 5B shows that motile filopodia accounted for most of the protrusions extended from the dendrites of young (9–11 DIV) neurons. With increasing time in vitro, however, the density of motile filopodia decreased while spine density increased. By day 16 in vitro, the majority of dendritic protrusions were stable, suggesting that they were composed mainly of dendritic spines. The complementary time courses of filopodium and spine densities evident in Figures 5A and 5B therefore seem consistent with the proposal that dendritic spines are formed by the stabilization of motile dendritic filopodia.

#### Spine Appearance Is Associated with the Appearance of Functional Synapses

The proposal that dendritic filopodia serve primarily to initiate synaptogenic contacts suggests that such contacts might restrict the motility of the contacting filopodium, perhaps as a first step in its transformation into a stable dendritic spine. Figure 5C shows average motility scores (defined as the average motility of all the dendritic protrusions of the neuron) of the same neurons of Figures 5A and 5B, plotted as a function of time in vitro. Figure 5D shows the synaptic densities (defined as the



**Figure 5.** Time Course of Dendritic Stabilization and Correlation with Synapse Formation

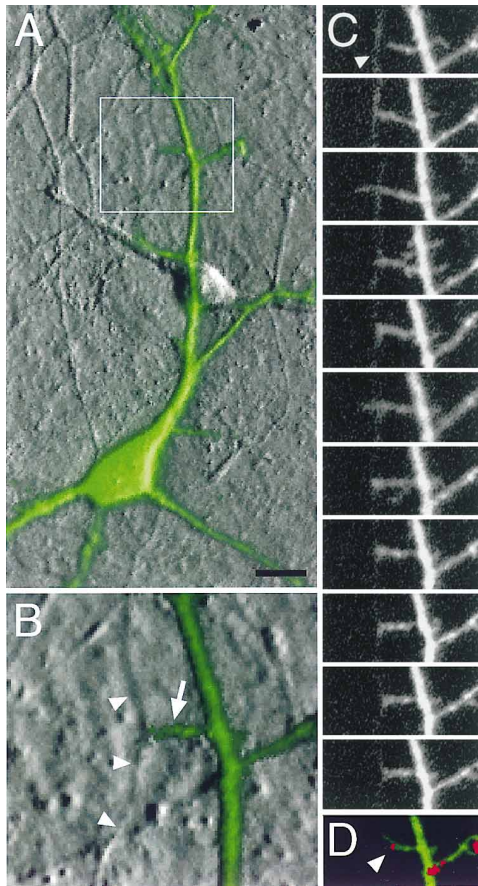
(A) Filopodium density at increasing times in vitro. Any dendritic protrusion whose motility score was greater than 1  $\mu\text{m}/3\text{ min}$  was considered to be a filopodium. Every point represents the 1 hr cumulative filopodium density of one neuron (as explained in Results). (B) Spine density at increasing times in vitro. Same neurons as in (A). (C) Changes in the average motility of dendritic protrusions. Every point represents the averaged motility score of all the filopodia and spines of one neuron observed for at least 1 hr. A large decrease in filopodium motility is observed around days 12–14 in vitro. Same neurons as in (A). (D) Time course of synapse formation. A large increase in the number of synaptic boutons formed per unit length of dendrite (synaptic density) is observed around days 12–14 in vitro. Same neurons as in (A). (E) Correlation of dendritic stabilization with synapse formation. The motility scores (C) plotted against synaptic densities (D) for the same neurons indicate that reduction in filopodia motility is correlated with an increase in synaptic density (Correlation coefficient equals  $-0.94$ ,  $P < 10^{-6}$ , Pearson product moment correlation test).

number of FM 4–64–labeled boutons per 100  $\mu\text{m}$  of DiO-labeled dendrite) for the same neurons. Up to day 11, very few functional synapses had formed on the dendrites examined in these experiments. However, a sharp increase in synaptic density occurred at days 12–14 in vitro, after which the synaptic density plateaued at approximately 25 boutons/100  $\mu\text{m}$  dendrite. This transition period appeared to coincide with the period during which a large decrease in dendritic protrusive activity was observed (Figures 5A and 5C), suggesting a direct relationship between these two parameters. Indeed, when the motility data of Figure 5C is plotted against the synaptic density data for the same neurons (Figure 5D), a significant inverse correlation between these parameters is seen (Figure 5E). Furthermore, the neurons examined seemed to fall into one of two categories: sparsely innervated neurons with highly motile dendritic protrusions or densely innervated neurons with stable dendritic protrusions. The segregation of the neurons examined here into two distinct groups may suggest that the transition from one state into another occurs quite rapidly and that any intermediate state is rather short-lived.

The reduction in dendritic protrusive activity observed around days 12–14 is followed by a period during which the rate of additional synapse formation is drastically reduced (Figures 5C and 5D). This further supports the idea that dendritic filopodia are instrumental in establishing axo-dendritic contacts. Interestingly, we have occasionally recorded somewhat higher synaptic densities (30–35 boutons/100  $\mu\text{m}$  dendrite) in very old (more than 45 DIV) preparations. Thus, synapses may continue to form at later periods (albeit at a very slow rate), suggesting that the decrease in additional synapse formation observed after day 16 in vitro does not result from a total loss of competence for synaptogenesis.

As previously noted, dendritic protrusions associated with a presynaptic bouton were, as a rule, persistent. This does not necessarily reflect a global condition of either the dendrite or the target cell as a whole. In neurons younger than 16 DIV, some (but not all) neighboring dendritic protrusions not associated with presynaptic boutons displayed relatively high motility levels (see, for example, Figure 7A). A quantitative comparison of the average motility levels of protrusions associated with presynaptic boutons (average motility =  $0.59\text{ }\mu\text{m}/3\text{ min}$ ) to those not associated with presynaptic boutons (average motility =  $1.30\text{ }\mu\text{m}/3\text{ min}$ ) supports this observation (6 neurons, 160 filopodia and spines, 11–15 DIV).

Conversely, even in neurons as old as 55 DIV, some stable dendritic protrusions did not appear to be juxtaposed to a functional presynaptic bouton. For example, in the older (more than 15 DIV) neurons portrayed in Figure 5, 36.2% of the stable protrusions had no apparent presynaptic partner (5 neurons, 84 spines). It is possible that the apparently absent presynaptic terminals simply were not detected by our functional assay. However, a similar finding was reported by Papa et al. (1995) who used antibodies against synaptophysin to stain presynaptic boutons. It is also possible that the presence of spines lacking presynaptic partners reflects ongoing processes of axonal degeneration and synapse elimination.



**Figure 6. Dendritic Filopodia Can Initiate Synaptogenic Contacts with Nearby Axons**

(A) A composite image of a FAST DiO-labeled neuron overlaid onto a DIC image of the same field.

(B) A high magnification of the region enclosed in a rectangle in (A). Note the axon (arrowheads) and the labeled filopodium (arrow) that spanned the gap between the dendritic shaft and the axon. Corresponds to the last timepoint in the time-lapse sequence shown in (C). (C) Time-lapse sequence composed of 11 frames at approximately 9 min intervals of a subregion of (B). A filopodium extended from the dendritic shaft and established a contact with the axon (arrowhead in the upper panel). Once the contact was established, the filopodium stayed attached to the axon for the duration of the time-lapse sequence.

(D) A composite image of the FAST DiO-labeled dendrite (green) and FM 4-64-labeled presynaptic boutons (red). Note the bouton that had formed at the contact site between the axon and the dendrite (arrowhead). Scale bar, 10  $\mu\text{m}$  (A).

#### Synapses May Be Found at Contact Sites between Dendritic Filopodia and Nearby Axons

The ability of dendritic filopodia to initiate synaptogenic contacts with nearby axons is directly demonstrated in Figure 6. In this experiment, a dendritic filopodium was observed to extend from the dendritic shaft and establish a contact with an axon lying at a distance of 7  $\mu\text{m}$  from the dendrite (Figures 6A and 6B). This filopodium was stabilized and stayed attached to the axon for the duration of the time-lapse session (Figure 6C). At the end of the experiment (90 min from the time of contact formation) FM 4-64 staining revealed the presence of a

presynaptic bouton at the filopodium contact site (Figure 6D). This nascent presynaptic bouton was rather small in comparison to older presynaptic boutons, but this is not unexpected for such newly formed presynaptic terminals (e.g., see Buchanan et al., 1989). Although this observation was anecdotal, it reinforces the idea that dendritic filopodia are capable of initiating synaptogenic contacts with neighboring axons.

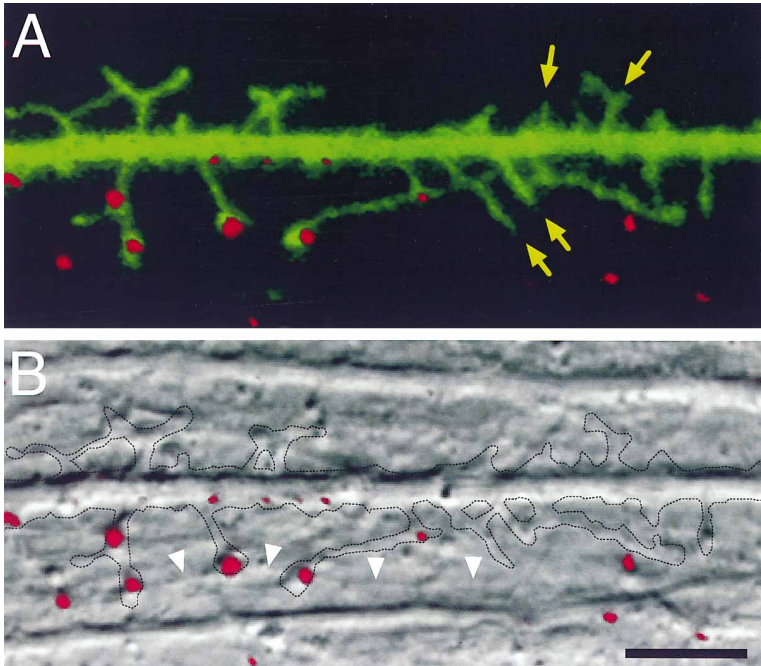
Careful examination of long dendritic spines associated with presynaptic boutons further supports the proposition that dendritic filopodia can initiate synaptogenic contacts with nearby axons. This is exemplified by Figure 7. This figure shows a FAST DiO-labeled dendrite of a 14 DIV neuron that had extended several long dendritic protrusions. FM 4-64 labeling revealed the presence of several presynaptic boutons positioned exactly at the sites where these protrusions contacted a nearby parallel axon observed in a DIC image of the same field (Figure 7B). How were the contacts between the axon and the parallel dendrite established? Given the protrusive capabilities displayed by dendritic filopodia and their ability to establish contacts with neighboring axons (see Figure 6), it seems most likely that the filopodia spanned the 5–7  $\mu\text{m}$  gap between the two neurites and that synapses subsequently formed at the contact sites.

#### Discussion

In the current study, we explored the possible involvement of dendritic filopodia in developmental synaptogenesis and spine formation. Using live cultured hippocampal neurons and vital fluorescent labeling methods, we showed that dendritic filopodia of immature dendrites exhibit vigorous protrusive behavior, consistent with roles in short-range (5–10  $\mu\text{m}$ ) exploration and contact initiation. We observed that synaptogenesis coincided temporally with a large reduction in the numbers of these motile filopodia and a concomitant increase in the numbers of dendritic spines. After filopodial motility reached its minimal level, there was a near cessation of additional synapse formation. Although the number of dendritic filopodia observable at any one time was much less than the number of spines seen on mature dendrites, analysis based on time-lapse observations suggested that the cumulative numbers of filopodia formed during development would greatly exceed the number of spines. Finally, we established that dendritic filopodia can initiate contacts with nearby axons that result, in some cases, in the formation of a presynaptic bouton. These observations are therefore consistent with the hypothesis that dendritic filopodia can actively initiate synaptogenic contacts with axons, and, at least in some cases, evolve into dendritic spines.

#### Possible Roles of Dendritic Filopodia in Synaptogenic Contact Establishment

The observations reported here were motivated by a working hypothesis that cast the dendritic filopodium in an active role during synaptogenic contact initiation. Many of these observations are also consistent, however, with a more passive role. That is, dendritic filopodia



**Figure 7.** Synapses at Sites of Contact between Filopodium-Like Dendritic Protrusions and Axons

(A) High magnification of a FAST DiO-labeled dendrite (green) and FM 4-64-labeled presynaptic boutons (red) in a 14 DIV preparation. (B) DIC image of the same field. Note that the parallel axon (arrowheads) 5–7  $\mu\text{m}$  below the labeled dendrite (dashed outline) has formed synapses (red) with several dendritic protrusions. Time-lapse analysis indicated that most of these dendritic protrusions displayed little change over time (data not shown). However, several filopodia (arrows in [A]) displayed levels of motility comparable to those observed along immature dendrites. Scale bar, 10  $\mu\text{m}$ .

could facilitate synaptogenesis simply by increasing the effective target area presented by a dendrite to ingrowing axonal growth cones. Of course, it is also possible that some dendritic filopodia actively initiate contacts while others are passive recipients. More information on the dynamics of axons during synaptogenesis will be necessary to assess whether dendritic filopodia play predominantly active or passive roles in synaptogenic contact.

Regardless of whether the role of dendritic filopodia in synaptogenic contact initiation is active or passive, these filopodia may be thought of as defining a “virtual dendrite,” that is, an extended volume within which a given dendrite can establish synaptic contact. In the active filopodium model, the protrusive filopodia would define a dendritic “capture volume.” In the passive model, these filopodia would define an expanded “target volume.” Interestingly, the volumes of adjacent virtual dendrites could overlap in ways that would be impossible for real physical dendrites and might thereby permit otherwise unlikely patterns of axo-dendritic interconnection.

The mechanisms that regulate filopodia extension are currently unknown. It has been proposed that dendritic filopodia may be extended in response to the neurotransmitter glutamate (Smith and Jahr, 1992), possibly secreted by approaching axonal growth cones. In the course of this study, however, we have observed that filopodium extension persists in the presence of the ionotropic glutamate channel blockers 6-cyano-7-nitroquinoxaline-2,3-dione, (CNQX, 10  $\mu\text{M}$ ) and D,L-2-amino-5-phosphonovaleric acid (AP-5, 50  $\mu\text{M}$ ; data not shown). Furthermore we have observed vigorous filopodial extension in the apparent absence of nearby axons (data not shown). Thus, while it remains possible that neurotransmitters may regulate filopodia in important ways, it is clear that glutamate-receptor channel activation is

not an absolute requirement for the extension of dendritic filopodia.

The increase in synaptic density that occurs around 12–14 DIV appears to coincide with a large reduction in the motility of dendritic protrusions (Figures 5C–5E). During this period, dendritic protrusions associated with presynaptic boutons are invariably persistent, while some protrusions, not associated with presynaptic boutons, retain their dynamic filopodium-like characteristics. At later times, however, almost all dendritic protrusions are persistent and relatively stable, including protrusions that do not seem to be associated with presynaptic boutons (see also Papa et al., 1995). We have not yet defined the causal relationships (if any) between the progress of synaptogenesis and the decline of filopodial motility. In fact, both processes could be regulated by a third, as yet undetermined developmental process (e.g., see Fletcher et al., 1994; Basarsky et al., 1994). It is nonetheless tempting to speculate on the interesting consequences that might arise from the regulation of filopodial motility by the density of synaptic connections formed on a dendrite. For example, if an increase in the number (or efficiency) of synapses formed on a dendrite could suppress the protrusive activity exhibited by the filopodia of that dendrite (as suggested by Figure 5E), the virtual dendrite defined by these filopodia would constrict, and as a consequence, the ability of that dendrite to establish additional synaptic contacts would be suppressed (as discussed above). Such a hypothetical regulatory mechanism could serve to balance the innervation density among tightly packed dendrites competing for the same pool of afferent axons. In fact, some support for this proposed mechanism may be found in our observation that the decline in dendritic protrusive activity is followed by a period during which the rate of additional synapse formation is drastically reduced (Figures 5C and 5D).



### Dendritic Filopodia May Be Developmental Precursors of Dendritic Spines

The notion that the dendritic filopodium is the direct and immediate precursor to the dendritic spine is appealing in its simplicity. We have accumulated three new lines of evidence in support of this idea: a reduction in filopodium density coincides closely with an increase in the density of synapse-bearing spines (Figures 5A, 5B, and 5D); filopodia are formed during development in numbers sufficient to account for the entire population of spines on mature dendrites; and dendritic filopodia can undergo a transition from dynamic filopodial motility to spine-like stability during which a presynaptic bouton is formed on the newly stabilized protrusion (Figure 6).

Strong arguments have also been made, however, for a very different model of spine development. Based on a careful ultrastructural analysis of spine morphology in hippocampal area CA1 of juvenile and adult rats, Harris and colleagues have suggested that shaft synapses, not filopodia, are the most likely precursors to the dendritic spine and its associated synapse (Harris et al., 1989, 1992; see also Juraska and Fikova, 1979; Miller and Peters, 1981; Steward et al., 1988; Dailey and Smith, 1996). This conclusion was based on their finding that the relative frequencies of synapses formed on different spine types differ in 7-day-old, 15-day-old, and adult rats: in 7-day-old rats, most synapses are found on dendritic shafts and some are found on stubby spines, but none are found on thin spines (Harris et al., 1989). In 15-day-old rats, stubby spine synapses compose the largest group of spine synapses, with many synapses found on thin spines. In the adult rat, most synapses are found on thin spines but relatively few on stubby spines (Harris et al., 1992). These findings seemed most readily interpretable by a sequential evolution of shaft synapses into stubby spine synapses into thin spine synapses (Harris et al., 1989, 1992). While this interpretation provides the most parsimonious interpretation of the ultrastructural findings, other interpretations remain possible.

One alternative interpretation might be that spines newly formed at different developmental stages assume different morphologies. Thus, the altered spine-type distribution observed in progressively older rats may result from ongoing synapse formation and elimination rather than the gradual remodeling of a given spine population. In fact, doublings of synaptic densities were observed from day 7 to day 15 (Harris et al., 1989) and from day 15 to adulthood (Harris et al., 1992). This suggests that a great deal of synapse formation (probably accompanied by some degree of synapse elimination) occurs in the rat hippocampus between day 7 and adulthood. Of course, it is also possible that spines are formed *in vivo* by more than one process, e.g., from shaft synapses evolving into spines, as proposed by Harris et al., and by dendritic filopodia transforming into spines, as proposed here. More work on the dynamics of synaptogenesis in tissues, perhaps along the lines of that begun by Dailey and coworkers (Dailey et al., 1994; Dailey and Smith, 1996), will be necessary to clarify this important issue.

The transient appearance of dendritic filopodia has been described *in vivo* even in neurons that do not have

spines in their adult form (Lund et al., 1977; Phelps et al., 1983; Ramoa et al., 1987; Saito et al., 1992). While this might appear to call the relevance of filopodia to spine formation into question, we would suggest that dendritic filopodia in such a setting may still play active roles in short-range contact initiation (Saito et al., 1992) but simply retract more fully during maturation than is the case in spine-synapse formation.

### Conclusions

The findings presented in this study strongly support the idea that dendritic filopodia and their protrusive motility play important roles in initiation of synaptogenic contacts between axons and dendrites in the mammalian CNS. The recognition that dendritic structures could be central to the initiation of synaptogenic contacts raises new and interesting possibilities regarding the mechanisms that underlie synapse formation, stabilization, and elimination. For example, the initiation of synaptogenic contacts by dendritic filopodia might provide a mechanism by which new axo-dendritic synapses may be established without a requirement for axonal growth cones. Such mechanisms may be particularly important in postdevelopmental synaptic remodeling processes, such as those sometimes postulated to underlie long-term memory. It is also worth noting that just as synapses may be formed by protrusive dendritic filopodia, they might also be eliminated by the detachment of such filopodia (or nascent spines) from their axonal counterparts. It is interesting to consider the possibility that activity-dependent processes may underlie the stabilization or elimination of such contacts: if dendritic filopodia evolve into dendritic spines, as proposed in our study, experimental treatments aimed at blocking activity-dependent synapse elimination would be expected to increase the density of dendritic spines. Indeed, this prediction is supported by several recent studies (e.g., Dalva et al., 1994; Rocha and Sur, 1995).

Dendrites possess a sophisticated repertoire of receptors, ion channels, and other signaling mechanisms that are generally not identified in axons (Craig and Banker, 1994). Furthermore, dendrites, but not axons, possess machinery for local protein synthesis. These unique properties suggest that dendrite-specific mechanisms may regulate dendrite-initiated contacts in ways that would seem to have no counterparts in axons. For instance, the involvement of N-methyl-D-aspartate receptors in synaptogenesis (Constantine-Paton, 1990) may suggest that N-methyl-D-aspartate receptors could positively or negatively regulate dendritic-based initiation of synaptogenic contacts (see also Smith and Jahr, 1992).

At present, many basic questions regarding the process of "wiring" the mammalian brain remain open. Major gaps in our knowledge arise from a paucity of data derived from observations in live tissue. In fact, most of the current understanding of this subject is based on structural studies of fixed specimens. We expect that the use of live cultured neurons and vital staining methods, such as those used here, will provide exciting new information on the processes that define and modulate the connectivity of CNS neurons in development.

## Experimental Procedures

### Cell Culture

Hippocampal cell cultures were prepared as previously described (Ryan et al., 1993). Briefly hippocampal CA1–CA3 regions were dissected from 3–4-day-old Sprague-Dawley rats, dissociated by trypsin treatment (10 mg/ml for 5 minutes at room temperature) followed by trituration with a siliconized Pasteur pipette, and then plated onto coverslips coated with Poly-D-Lysine (Sigma) inside microwells formed by sealing a 6 mm diameter glass cylinder (Bellco Glass) onto the coverslip using silicone sealant (Dow-Corning). Culture media consisted of minimal essential media (Gibco), 0.6% glucose, 0.1 g/l bovine transferrin (Calbiochem), 0.25 g/l insulin (Sigma), 0.3 g/l glutamine, 5–10% FCS (Hyclone), 2% B-27 supplement (Gibco) and 8  $\mu$ M cytosine  $\beta$ -D-arabinofuranoside. Cultures were maintained at 37°C in 95% air 5% CO<sub>2</sub> humidified incubator, and culture media was replaced every 3–4 days.

### Labeling Neurons with FAST DiO

Cells were examined 6–28 days after plating. The coverslips on which the cells were grown were mounted on the microscope in an open laminar perfusion chamber and superfused constantly at a rate of approximately 1 ml/min with a saline solution consisting of 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 25 mM HEPES (buffered to pH 7.4), and 30 mM glucose. The stage was heated continuously with a stream of air heated to 37°C, resulting in a chamber temperature of 30–32°C. A solution of FAST DiO (3,3'-dilinoleyloxycarbocyanine perchlorate), which is a diunsaturated linoleyl analog of DiO (Honig and Hume, 1986, 1989) was prepared by mixing one part of FAST DiO (Molecular Probes) from a stock solution of 5 mg/ml in dimethyl formamide, with 200 parts of a filtered carrier solution consisting of 0.35 M sucrose in distilled water. Glass micropipettes (tip diameter 2–4  $\mu$ m) were filled with the FAST DiO solution, mounted on a hydraulic micromanipulator (Narashige), and positioned just above the soma of the neuron about to be labeled. The dye solution was ejected by applying brief (25 ms) pulses of pressurized air to the micropipette with a picospritzer (General Valve). The labeling intensity was monitored by fluorescence microscopy during the labeling procedure, and the micropipette was removed after an adequate degree of labeling was achieved (Figures 1A and 1D). The chamber was then covered with a second coverslip, confining the volume of the chamber to approximately 90  $\mu$ l.

### Functional Labeling of Presynaptic Boutons with FM 4-64

Functional presynaptic boutons in the field were visualized by selectively loading them FM 4-64 (N-(3-triethylammoniumpropyl)-4-(6-(diethylamino)phenyl)hexatrienyl)pyridinium, dibromide; Molecular Probes). FM 4-64 (Henkel and Betz, 1995) is similar in structure and properties to FM 1-43, a dye used to label synaptic vesicles selectively in live neurons (Betz and Bewick, 1992, 1993; Ryan et al., 1993, 1995), but its longer wavelength emission spectra make it more suitable for dual-channel fluorescence microscopy in conjunction with green fluorescent dyes, such as DiO.

Cells were exposed to FM 4-64 by replacing the saline in the perfusion chamber with saline containing 15  $\mu$ M of FM 4-64. The neurons were then stimulated to fire action potentials by passing brief (1 ms) current pulses through the chamber via agar bridges affixed to opposite sides of the chamber, into which Ag–AgCl electrodes were inserted (see also Ryan et al., 1995). The cells were stimulated for 60 s at 10 Hz, left in the presence of the dye for an additional minute, and then rinsed with saline for 5–10 min. After collecting a digital image of the labeled field (such as that in Figure 1B), the cells were stimulated again for 90 s at 10 Hz, in order to unload the dye previously taken up by synaptic vesicles, and a second image of the same field was collected (Figure 1C). The spatial distribution of the presynaptic boutons was determined from a difference image obtained by subtracting the second image from the first one, which was then digitally overlaid onto the fluorescence image of the FAST DiO-labeled neuron (Figure 1D).

### Microscopy

Scanning fluorescence and DIC images were acquired using a modified Biorad MRC 500 laser scanning unit coupled to a Zeiss IM-35

inverted microscope equipped with a Nikon 40  $\times$  1.3 N.A. Fluor objective. The cells, labeled with DiO and FM 4-64, were excited using the 488 nm line of an argon laser. A 565 nm dichroic mirror was used to split the emitted light between two channels, and the longer wavelength light was passed through a 630 nm long pass filter. This optical configuration resulted in a clean separation of the fluorescence signals arising from the two fluorescent dyes used in this study, with practically no crosstalk between channels. DIC images were acquired by collecting the transmitted excitation light with a third detector and Zeiss DIC optical components.

### Time-Lapse Recordings and Data Analysis

Time-lapse recordings of FAST DiO-labeled dendrites were carried out for 60–90 min, during which time images were collected at a rate of one image every 3 min. Digital images were created by averaging 4–10 frames collected at a single focal plane with the confocal apertures fully open at a resolution of 768  $\times$  512 (8 bits/pixel). At the end of the time-lapse session, the spatial distribution of presynaptic boutons was determined by FM 4-64 staining, as described above. Finally, another image of the FAST DiO-labeled dendrites was collected, in order to correct for slight translation movements that may have occurred during the FM 4-64 labeling procedure.

The lengths of individual filopodia and spines were determined manually by means of a mouse-driven imaging software package written for this purpose. The length of a filopodium or spine was defined to be the linear distance between its tip and its base. The motility score for any given filopodium or spine was defined as the average of absolute values of changes in its length between consecutive images over the entire time-lapse session. In many cases, the length of a filopodium decreased during the time-lapse sequence to the point that it was no longer discernible from the dendrite shaft. Therefore, only timepoints in which the length of a filopodium or spine exceeded 0.6  $\mu$ m in one or both of two consecutive images were included in the data set used to calculate the motility of this filopodium or spine. Conversely, if the length of a spine or filopodium was less than 0.6  $\mu$ m in two consecutive images, the timepoint was excluded from the motility calculation. Spines or filopodia whose length was reduced below 0.6  $\mu$ m were considered to be 0  $\mu$ m in length.

For each time-lapse experiment, the synaptic density (defined as the number of presynaptic boutons formed on 100  $\mu$ m of FAST DiO-labeled dendrite), was determined as follows: the FM 4-64 difference image generated at the end of the experiment was passed through a 3  $\times$  3 median filter and digitally overlaid onto the last image of the FAST DiO-labeled dendrites collected during the time-lapse session. Single fluorescent puncta that appeared to be in direct contact with shafts or spines of the labeled dendrites were counted, and this number was divided by the total length of all the labeled dendritic segments in the field.

### Avoidance of Phototoxicity Effects

Phototoxic damage was minimized by labeling with minimal quantities of FAST DiO. Under these conditions, no morphological or physiological changes attributable to phototoxic damage were ever detected. In contrast, when neurons were mistakenly labeled with larger quantities of FAST DiO, they almost invariably showed visible signs of phototoxic damage; i.e., blebbing of fine dendrites or retraction of filopodia. We also tested for less obvious cumulative photodamage by plotting the average lengths of all filopodia and spines included in the study as a function of time from the beginning of the experiment. However, this analysis showed no consistent time-dependent trend in the average filopodium and spine length (data not shown). While this does not completely exclude some undetected deleterious effects of recurrent illumination, it suggests that the magnitude of these effects was, at most, very small.

### MAP2 Staining

Neurons were fixed on the microscope stage by flooding the perfusion chamber with a fixative solution consisting of 4% formaldehyde and 120 mM sucrose in phosphate-buffered saline (PBS) for 30 min. The cells were permeabilized for 10 min in fixative solution to which 0.2% Triton X-100 was added. The cells were washed three times

in PBS, incubated in 10% bovine serum albumin for 1 hr at 37°C, rinsed briefly with PBS, and incubated overnight at 4°C with a primary monoclonal antibody against MAP-2 (Sigma, clone HM-2) diluted 1:250 in PBS and 1% bovine serum albumin. The cells were then rinsed three times for 10 min with PBS and incubated for 1 hr at room temperature with a fluorescein-conjugated goat anti-mouse secondary antibody (Sigma). The cells were rinsed again with PBS, mounted, and imaged immediately.

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