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Analysis of Spine Morphological Plasticity in Developing Hippocampal Pyramidal Neurons

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ABSTRACT: Dendritic spines are targets of most excitatory inputs in the central nervous system (CNS) and are morphologically heterogeneous. Ultrastructural studies have traditionally classified spines into four major categories (filopodia, stubby, thin, and mushroom) based on their distinct morphologies. The recent discovery of rapid morphological plasticity of spines has raised the possibility that those categories, rather than being intrinsically different populations of spines, represent instead temporal snapshots of a single dynamic phenomenon. We examined this question with two-photon time-lapse imaging of developing hippocampal pyramidal neurons, transfected with E-GFP in cultured slices. After blind scoring to morphologically classify spines into the four traditional groups, we analyzed the fate of populations of spines over a period of 2–4 h. We found considerable morphological conversions among all categories, although systematic trends were detected. While most stubbies and spines (defined for our analysis as the combination of thin and mushroom protrusions) retained their basic morphologies, most filopodia transformed into stubbies and spines, although they could also extend out of existing spines. Our results suggest that in developing hippocampal pyramidal neurons, traditional morphological distinctions are stable over short (<4 h) periods of time, but that at the same time, considerable mixing among these groups takes place. *Hippocampus 2000;10:561–568.*
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INTRODUCTION

Dendritic spines are morphological specializations present in many mammalian neurons, specialized for receiving synaptic inputs (Harris and Kater, 1994; Shepherd, 1996). Over a century ago, Ramón y Cajal (1888) first pointed out that dendrites had small thorns resembling leaves from a tree, or spines from a rose stem. He called them "espinas" in the original Spanish text, which was published as the first article in the first issue of a journal that he himself financed. Later, he generalized the term to similar structures found throughout the vertebrate central nervous system (CNS) (Ramón y Cajal, 1891b, 1904). It was also Cajal who first speculated about the function of spines with two key hypotheses: 1) that spines connect axons and dendrites (Ramón y Cajal, 1891a, 1894), and 2) that they are involved in learning (Ramón y Cajal, 1893). In the 1950s the introduction of electron microscopy (EM) enabled investigators to explore the structure of dendritic spines and synapses with unprecedented detail. Cajal's neuronal theory was proven correct by DeRobertis and Bennett (1955) and Palay (1956), who in two landmark studies demonstrated the presence of synapses in a variety of preparations. Shortly afterwards, Gray (1959) identified synapses on dendritic spines.

Spines have different shapes, and these different morphologies could be related to different developmental or functional roles. Researchers interested in spines have traditionally followed the morphological classification of spines suggested by Peters and Kaiserman-Abramof (1970). They classified spines into three groups: *thin*, characterized by a bulbous head and attached to the dendrite by a fine neck; *mushroom-shaped* spines with broader heads; and *stubby* spines lacking a neck. A similar classification had been suggested by Jones and Powell (1969), where "sessile" and "pedunculated" referred to stubby and mushroom or thin spines, respectively. In addition, in developing neurons, researchers identified *filopodia*, which are typically longer, thinner processes than spines. Filopodia do not end in a bulbous head and based on this, and their length, are considered structurally unique from dendritic spines (Purpura, 1975; Fiala et al., 1998). Defining their functional characteristics is therefore of considerable interest. These four categories appear to capture well the range of diversity of dendritic protrusions and consequently have been widely used (e.g., Harris et al., 1992).

In recent years, the introduction of live imaging approaches has provided evidence that the morphology of dendritic spines is dynamic. Imaging of dendrites from hippocampal pyramidal neurons in dissociated culture or in brain slices has demonstrated that filopodia are remarkably dynamic and might be precursors of spines (Ziv and Smith, 1996; Dailey and Smith, 1996). Also, filopodia and spines can be formed in response to synaptic stimulation (Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999). In addition, rapid spine motility and morphological plasticity have been documented in dissociated cultures and acute brain slices from the hippocampus, neocortex, and cerebellum (Fischer et al., 1998; Kaech et al., 1999; Dunaevsky et al., 1999). In these

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studies, most spines undergo dramatic changes in shape in periods of time as short as a few seconds. Based on these data, we previously raised the possibility that morphological classifications of spines do not represent intrinsically different types of spines, but instead represent shapes that a single spine can transit through its lifetime (Dunaevsky et al., 1999). An alternative scenario is one where, although spine motility could still be prevalent, spines might not change into a different shape, and the four traditional morphological classifications could still hold.

To study this issue and better assess the dynamism of spine morphologies, we characterized whether there is any consistency in the shape of individual spines over time. We carried out two-photon time-lapse imaging of populations of dendritic protrusions from hippocampal pyramidal neurons in slices over periods of up to 2–4 h. We then scored blindly the morphologies of all the protrusions at each time point according to the traditional categories, and inquired whether protrusions that are classified in one category retained a similar morphology with time, or whether there was significant drift between protrusion morphologies. We encountered widespread mixing among all categories, although most stubbies and spines remained in the same category. Finally, we found that filopodia are particularly prone to become stubbies and spines, or emerge from them.

MATERIALS AND METHODS

Slice Cultures

All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1987). Hippocampal brain slices were harvested from C57 mice at age P0 to P1, using autoclaved dissection tools and 0.22-μm filtered solutions. The mice were cryoanesthetized on a bed of ice for 10 min, scrubbed with 70% ethanol, and decapitated with scissors. Working in a tissue culture hood, skin and skull were carefully cut along the dorsal cranial fissure with scissors and forceps and parted sideways. The brain was removed and placed into a fresh tissue culture dish filled with cold HEPES-artificial cerebrospinal fluid (HEPES-ACSF; 10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 5mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM glucose). Under a dissection microscope, the cerebral hemispheres were separated with a no. 15 surgical blade and oriented such that the diencephalon faced down. The cerebellum and mesencephalon were carefully dissected; after the hemisphere was rotated such that its medial side faced up, the diencephalon was removed with a surgical blade and a flat-ended spatula. The remaining piece of tissue, representing the cortex and hippocampus, was trimmed into a rectangular block with one edge parallel to the dorsal cranial fissure. Before use, the tissue chopper (TC-2 Tissue Sectioner, Smith & Farquhar) was sterilized with 70% ethanol inside the tissue culture hood, and the rectangular block of tissue was positioned for a parasaggital chopping orientation. The tissue was cut into 300-μm-thick slices. Using a flat-ended spatula, the slices were transferred to a fresh culture dish containing cold HEPES-ACSF. They were separated from each other with a surgical blade as soon as possible. Slices were mounted on 0.4-μm culture inserts (Millipore, PICM ORG 50). Slices were cultured in culture medium, composed of 50 ml Basal Medium Eagle (Gibco), 25 ml Hank's Balanced Salt Solution (Gibco), 25 ml horse serum (Hyclone), 0.65 g dextrose, 1.0 ml HEPES (Gibco), and 1.0 ml 100 × Pen-strep (Gibco). Three to six slices were placed on each insert, and the insert was transferred into a six-well culture plate, in which each well contained exactly 1 ml culture medium. Plates were then incubated for up to 2 weeks (5% CO_2, 37°C). Media were changed every other day.

Particle-Mediated Gene Transfer

Hippocampal slice cultures were transfected with the Helios Gene Gun System (Bio-Rad), following Lo et al. (1994) and Arnold et al. (1994). E-GFP-C1 (Clontech) was purified on a Qiagen column and precipitated onto the gold microcarrier particles according to the Helios Gene Gun instructions, with the exception that polyvinylpyrrolidone was omitted. The DNA loading ratio was 8 μg/mg gold. Tubing sets were stored with desiccant at 4°C. Slices were transfected after a minimum of 6–9 days in vitro (DIV). All the operations of the Gene Gun System followed the instruction manual. In order to reduce the damage of slices caused by high-pressure helium flow (T.W. Smith Corp.), we covered the tip of a barrel liner with a nylon mesh (Small Parts, Inc., ϕ90 μm) held with tape. The empty cartridge holder and barrel liner were kept under ultraviolet light for 20 min before loading. We adjusted the helium pressure to 150 psi. Two to three "preshots" were fired with an empty cartridge holder to clean the helium pathway and the mesh. We then removed the cover of the culture plate, held the gun perpendicular to the plate (with a distance of 1 cm between the tip of the barrel liner and the insert), and fired the gun. We covered the plate and cultured the slices as soon as possible under the same conditions.

Two-Photon Microscopy

Images were taken 2 days after transfection. Imaging was carried out at 37°C in a submerged chamber perfused with ACSF, with a custom-built two photon laser scanning microscope (Majewska et al., in press). The microscope consisted of a modified Fluoview (Olympus, Melville, NY) confocal microscope with a Ti:sapphire laser providing 130 fs pulses at 75 MHz at wavelengths of 740–850 nm (Mira, Coherent, Santa Clara, CA) pumped by a solid-state source (Verdi, Coherent). A 40×, 0.8-NA water immersion objective (IR1, Olympus) was used. Fluorescence was detected with photomultiplier tubes (PMTs; HC125-02, Hamamatsu, Japan) in external, whole-area detection mode, and images were acquired and analyzed using Fluoview (Olympus) software. Images of spines were taken at the highest digital zoom (×10), resulting in a nominal spatial resolution of 20 pixels per μm. Time-lapse series were acquired at intervals ranging from 10–30 min. At each time point, several focal planes 1 μm apart were imaged.

Image Analysis

Image analysis was done with NIH-Image in Macintosh computers. The individual images from the time-lapse series were temporally shuffled by one observer and were than analyzed by a different observer who was blind to the origin of the image. The analysis consisted of visually identifying all dendritic protrusions on every image and classifying their morphologies into one of four different categories according to traditional guidelines (see Results). For each sequence, the scoring of all the spines was done blindly, i.e., the observer classifying the spines was not aware of the time-point at which the frame was taken. Given the high number of spines present per frame, the observer also did not know whether a particular protrusion corresponded to any other protrusion from a different frame. After scoring every spine in all the images, the code was broken and, for each protrusion, a time series of categories was compiled, and prospective (forwards in time) and retrospective (backwards) analysis was carried out regarding the fate of the protrusion (see below). In the retrospective analysis, the numbers of detectable protrusions that remained within a category or switched categories was the same, but the number of undetectable protrusions changed, changing the overall percentages for each category.

RESULTS

Morphological Classification of Spines From Two-Photon Images

We analyzed 6 two-photon time-lapse sequences taken from 5 pyramidal neurons in hippocampal slice cultures from C57 mice (Fig. 1A). Slices were made at P0–1 and were kept in culture for 9–12 days. At these ages, both in cultured and acute slices, filopodia were still present and thus the four types of dendritic protrusions could be observed. Neurons were transfected with E-GFP driven under the CMV promoter 2 days before imaging. Two of the neurons were located in the CA1 region, two were in the CA3 region, and one was located in the CA2 region. No apparent differences were encountered between the neurons, and therefore we pooled the data. The imaged dendritic segments corresponded to secondary and tertiary branches from the apical dendritic tree, located in the stratum radiatum, 50–150 μm from the soma (Fig. 1B). Approximately 15–50 spines were present in each frame.

A total of 1,433 protrusions in 53 frames was classified as filopodia, stubby, thin, or mushroom (Fig. 2), according to traditional criteria (Peters and Kaiserman-Abramof, 1970; Purpura, 1975; Miller and Peters, 1981; Harris and Stevens, 1989; Harris et al., 1992; Fiala et al., 1998). Specifically, protrusions were classified as "filopodia" if they were relatively long and thin and did not exhibit a bulbous head (Fiala et al., 1998). "Stubby" spines did not have a clear neck, but were closely connected to the shaft of the dendrite, i.e., the diameter of the head was similar to the total length of the spine (Harris et al., 1992). "Thin" spines were identified as those protuberances that had long, thin necks (neck length much larger than its diameter), but had clear bulbous heads (diameter of the head larger than diameter of the neck; Harris et al., 1992). "Mushroom" spines were similar to "thin" spines, but were characterized by larger heads, i.e., the diameter of the head was much larger than diameter of the neck (Harris et al., 1992). Seven protrusions were difficult to classify and were not included in the analysis.

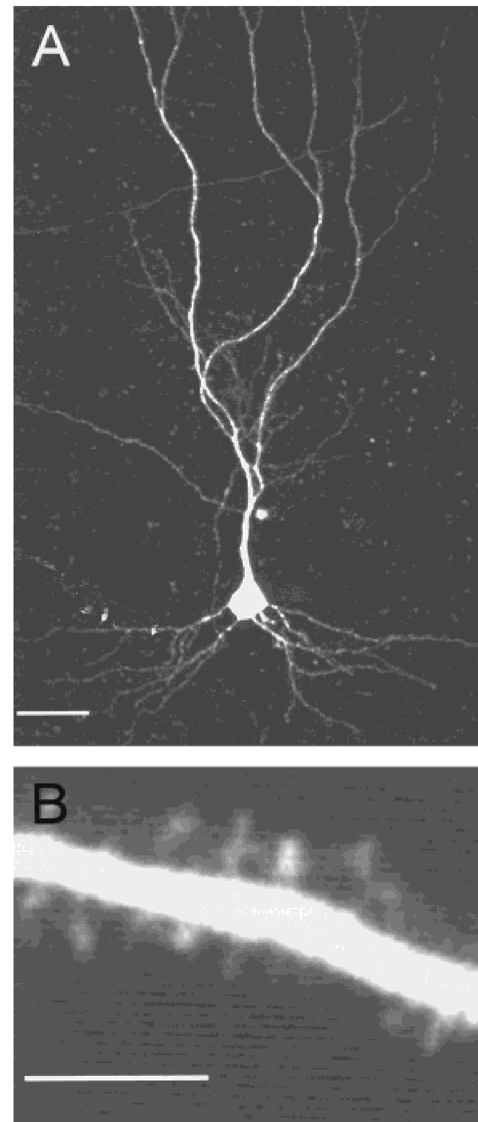

FIGURE 1. Two-photon imaging of living spines in transfected neurons. A: Two-photon image of a CA1 pyramidal neuron in cultured hippocampal slice. The slice was made at P0 and cultured for 9 days. The neuron was then transfected with E-GFP, using particle-mediated gene transfer, and incubated for another 2 days. Note how clearly the dendritic tree is discriminated over the background. Scale bar, 30 μm. B: Higher magnification of the apical dendritic tree of a similar neuron. Note how many different protrusions are clearly visible. Scale bar, 5 μm.

Prospective Analysis of Time-Lapse Sequences

In order to find out whether spine morphologies were constant over time, we individually characterized the temporal evolution of

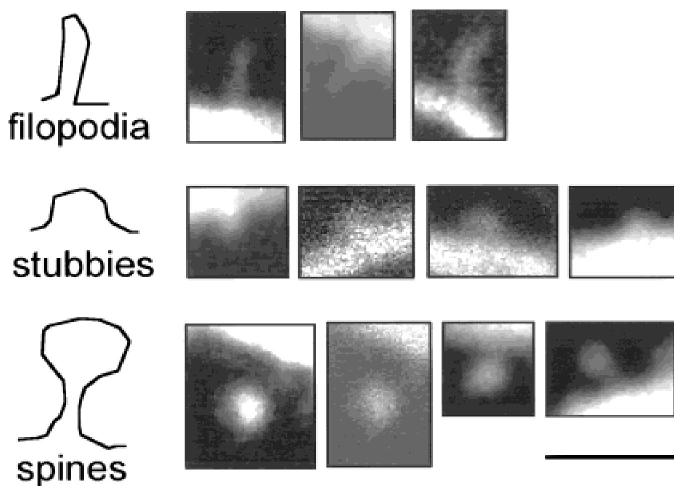

FIGURE 2. Classification of dendritic protrusions. Representative examples of two-photon images of protrusions categorized as filopodia, stubbies, or spines. The "spine" category encompasses both thin and mushroom spines. Scale bar, 3 μm. Note how the signal-to-noise ratio of the images is adequate for a morphological classification of the protrusions.

spine morphologies, assembling for each spine the categories in which it had been scored at every frame during the sequence. Due to the low number of mushroom spines (n = 6) and the subjective differentiation between mushrooms and thins, for the rest of the study we decided to merge thins and mushrooms into a new category called "spines." In this respect we followed the same convention as Fiala et al. (1998) and the original intuition of Jones and Powell (1969) to classify both groups together as "pedunculated." The low number of mushroom spines found in our sample may be explained by the developmental stage of the imaged cells: indeed, in hippocampal pyramidal cells, mushroom spines have not established their typical ultrastructural features until after P15 (Harris et al., 1992; Fiala et al., 1998).

To find which protrusion corresponded to which other protrusion in different frames, we used acetate sheets to mark the positions of dendritic landmarks and individual protrusions and played back the sequences at several speeds and alternated their displays. Although many different forms of spine motility have been observed by ourselves and others (Fischer et al., 1998; Dunaevsky et al., 1999), we have never detected translations of the position of the spine along the dendrite. Therefore we reasoned that we could use the exact position of spine in the dendrite to define the identity of each protrusion. For most protrusions, a precise correspondence could be found, i.e., a protrusion was present in the same exact position in subsequent frames. Nevertheless, we frequently found that a particular protrusion was clearly visible in one frame, yet could not be discerned in the subsequent or previous images and had therefore not been scored previously. Because of the limited spatial resolution of light microscopy and signal-to-noise limitations for very small structures, it is possible that those protrusions did not disappear but were instead very small. We therefore classified those protrusions as "undetectable" (32/156 protrusions).

To assess morphological plasticity at two different time scales, we carried out a prospective analysis of the protrusions during the 2–4-h sequences, and thus investigated the fate of every protrusion during the experiment (Fig. 3). We observed every possible combination of transitions among categories in the scored morphologies, although certain trends could be established. Protrusions scored as filopodia at the beginning of the sequences were mostly scored as stubbies (32%) or spines (35%) at the end. Only 16% of the original filopodia still scored as filopodia after 2–4 h. At the same time, most spines scored as stubbies at the beginning of the sequence were still considered stubbies at the end (54%), and most spines were also considered spines 2–4 h later (58%). A small percentage of stubbies (10%) were scored as spines at the end of the sequences, and also some spines (17%) were scored as stubbies at the end. Finally, a sizable number of protrusions from every category became undetectable at the end of the sequence. This was more likely to happen with protrusions originally scored as stubbies (30%), but was found also to occur with filopodia (16%) or spines (12%).

We conclude that over short periods of time (<4 h), the morphological classifications were maintained since, with the exception of filopodia (see below), protrusions were consistently categorized in the same groups during the sequence. At the same time, many protrusions (~70% of filopodia, ~15% of stubbies, and ~30% of spines, not including undetectables) were scored differently at the beginning and the end of the sequences, suggesting that considerable mixing among categories occurs.

Retrospective Analysis

In our prospective analysis we encountered many cases of protrusions that were undetectable at the end of the time-lapse sequence (32 protrusions; Fig. 3). These protrusions might have disappeared or become too small to detect. In particular, many stubbies (~30%) were scored as undetectable at the end of sequences, consistent with the possibility that the smallest stubbies were undetectable. At the same time, we found a slightly higher number of protrusions at the end (165) than the beginning of sequences (156). This suggested that protrusions present at the end of sequences and not detected at the beginning could represent newly formed spines or filopodia, which is expected in these developing neurons. To investigate this issue and explore the origin of all protrusions found at the end of sequences, we carried out a retrospective analysis of the data (Fig. 4).

Indeed, we found 41 protrusions that were scored as undetectable at the beginning of sequences. Most of them (33%) were scored as stubbies at the end of the sequence, whereas a sizable number were scored as filopodia (26%) or spines (12%). The flow in the prospective and retrospective analyses between stubbies and undetectables suggests that many undetectable protrusions are small stubbies. At the same time, the fact that clear filopodia and spines can be seen in locations where no protrusions were previously detected suggests that they were formed during the experiment.

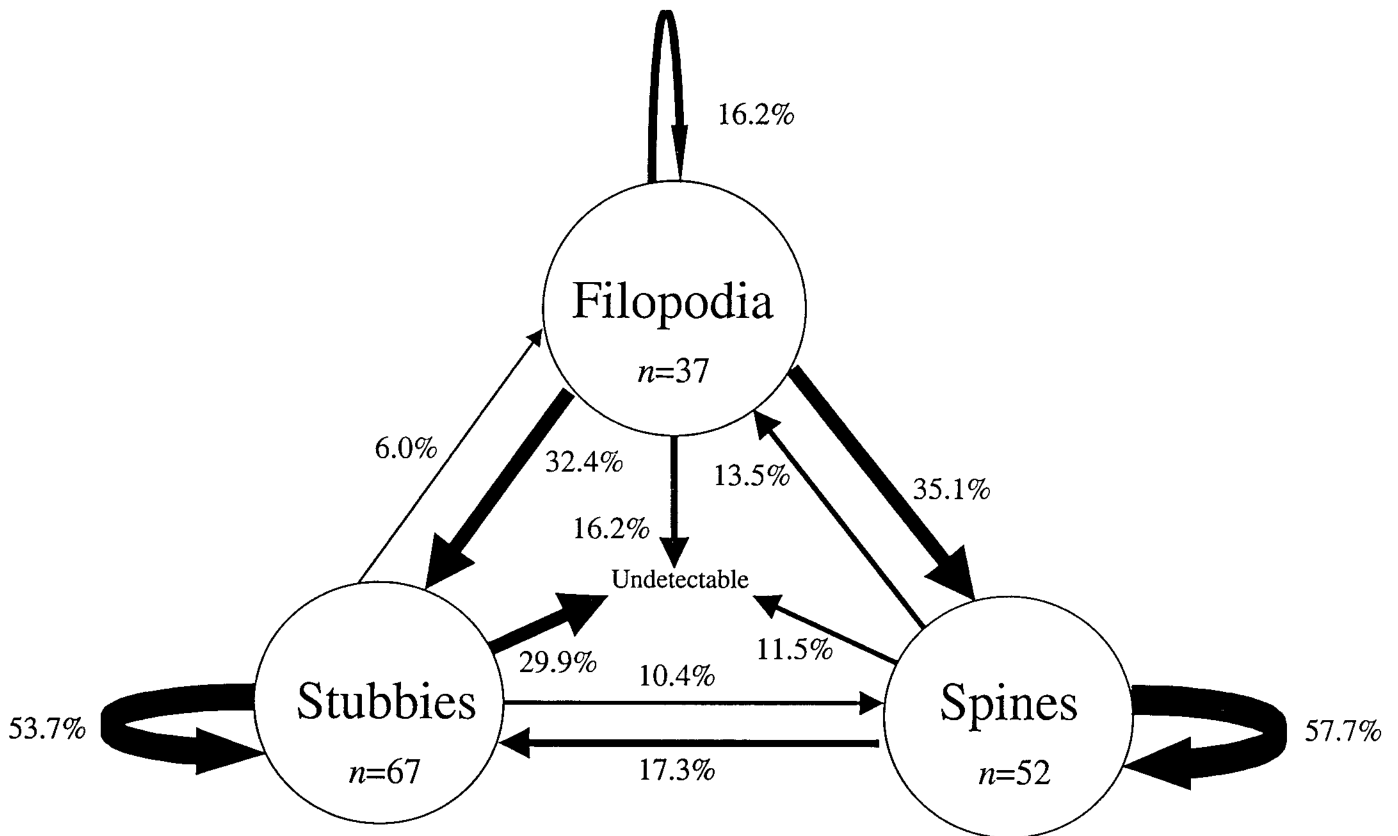

FIGURE 3. Prospective analysis of morphology of dendritic protrusions. Summary of results of the prospective (forwards in time) analysis. Each circle represents the population of protrusions categorized as filopodia, stubbies, or spines *at the beginning* of the time-lapse sequences. Arrows indicate the class in which those protrusions were categorized *at the end* of the 2–4-h time-lapse sequences. Thickness of a line is proportional to the percentage of the initial protrusions. Note how, in contrast to filopodia, most stubbies and spines are still classified in the same groups at the end of sequences.

Filopodia Are Dynamic

As indicated, in the prospective analysis, protrusions scored as filopodia at the beginning of sequences were likely to be classified as stubby or thin spines at the end. This special behavior suggested that they might represent precursors of these other structures. This idea has been proposed based on the developmental correlation between the disappearance of the filopodia and the appearance of these more mature spine forms (Ziv and Smith, 1996; Dailey and Smith, 1996; Papa et al., 1995). This would imply that there is a developmental sequence by which filopodia formed de novo and then turn into stubbies or spines. We tested this hypothesis with our retrospective analysis, inquiring about the origin of the filopodia found at the end of sequences. We found that although some filopodia had indeed been originally scored as undetectable (26%) in the beginning, most filopodia had originally been scored as stubbies (17%) or spines (30%), and only 26% of the filopodia were originally scored as filopodia. These data argue against a simple interpretation of the fate of the filopodia, and indicate that there is considerable back-and-forth conversion between filopodia and other forms of protrusions. Because filopodia are more likely to switch classes than other protrusions, they appear to be particularly plastic or labile. These results indicate that filopodia are very dynamic and are likely to convert to or emerge from other forms of spines.

DISCUSSION

We undertook this study to inquire whether different types of spine morphologies are stable over time. This issue has become pertinent because of recent reports of rapid spine motility in many spiny neurons (Fischer et al., 1998; Dunaevsky et al., 1999) and of emergence of filopodia or spines in response to synaptic stimulation (Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999). Large differences in the shape and size of spines are evident throughout the CNS and have been widely used as a guide to classify spines (Peters and Kaiserman-Abramof, 1970; Purpura, 1975; Harris and Kater, 1994). Indeed, the fact that a major function of spines, that of calcium compartmentalization, is regulated by spine morphological parameters such as spine neck length (Korkotian and Segal, 1998; Majewska et al., 2000) demonstrates that different spine morphologies are likely to have a functional relevance (Yuste et al., 2000), and that different types of spines could therefore carry out different functions.

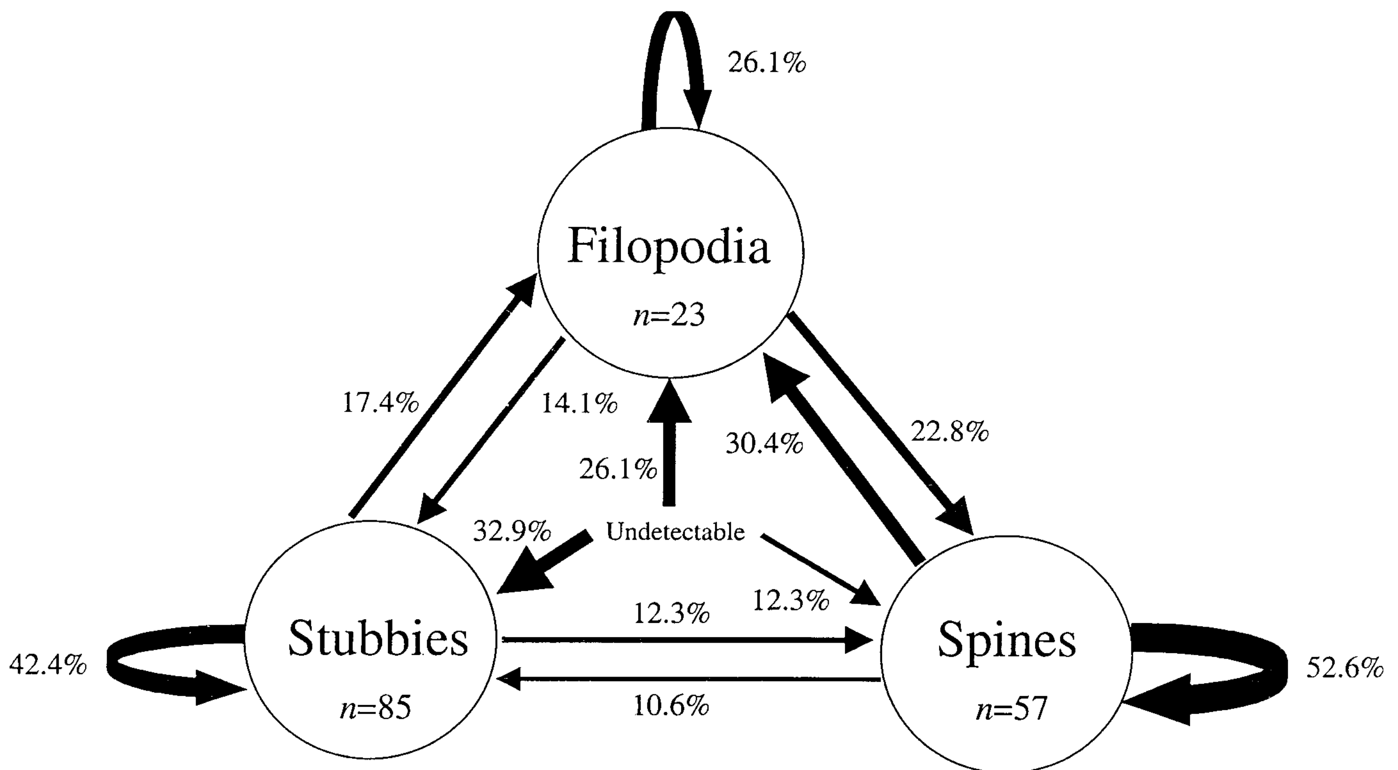

FIGURE 4. Retrospective analysis of the morphology of dendritic protrusions. Summary of the retrospective analysis (backwards in time). Circles represent protrusions *at the end* of the time-lapse sequences; arrows originate from the class in which those protrusions were categorized *at the beginning* of sequences. Note how most spines are classified as spines at the beginning of sequences, whereas most filopodia are scored as stubbies or spines. Meanwhile, stubbies are likely to be scored as stubbies or undetectable at the beginning of a sequence.

We limited our study to postnatal developmental stages (P0–1 + 9–12 DIV) for methodological and conceptual reasons. Hippocampal neurons in cultured slices can be optimally transfected with E-GFP and display a large variety of spine morphologies, including filopodia, which are very infrequent at older stages. Also, cultured slices display a similar range of rapid motility as acute slices, suggesting that developmental maturation in vitro and in vivo is proceeding at a similar rate (Dunaevsky et al., 1999). Finally, spine motility, which is developmentally regulated, involves nearly all spines in hippocampal neurons at these ages (Dunaevsky et al., 1999). Because of this, our conclusions are limited to these stages of development and may not necessarily apply to more mature hippocampal neurons.

Methodological Considerations

For this study, we took advantage of the enhanced depth penetration and reduced photodamage of two-photon excitation (Denk et al., 1990; Yuste and Denk, 1995) to carry out high-resolution imaging of spines in brain slices for periods of up to 4 h. We also used E-GFP transfections of neurons with particle-mediated gene transfer (Arnold et al., 1994; Lo et al., 1994), a method that fills living cells with high concentrations of GFP, a fluorophore that undergoes relatively little bleaching. Nevertheless, there are several potential limitations to this approach that are relevant for our conclusions. First, we examined the spine morphology of neurons from cultured brain slices, which may behave differently from neurons in behaving animals. Both the culturing and/or the slicing procedure could conceivably have an important effect on the morphological plasticity of spines. Although imaging of spine motility in vivo has been carried out in anesthetized preparations (Lendvai et al., 2000), it still has not been attempted in awake animals, and volatile anesthetics can interfere with spine motility (Kaech et al., 1999). In this respect, the development of low-weight, portable two-photon microscopes (Helmchen, 1999) and in vivo transfection of neurons with particle-mediated gene transfer (Tashiro and Yuste, unpublished findings) or viral vectors (Lendvai et al., 2000) could enable experiments designed to determine the degree of spine morphological plasticity in awake preparations.

Another potential problem with our experiments is that the population of spines may not be representative of the spines present in these cells. Using fluorescence recovery after photobleaching, we measured very fast (<100 ms), monoexponential spine diffusional equilibration times for E-GFP in our transfected neurons (Majewska et al., 2000). This indicates that cytoplasmic GFP will quickly diffuse into new protrusions and faithfully track morphological spines rearrangements that occur

over much slower (seconds to minutes) time periods (Fischer et al., 1998; Dunaevsky et al., 1999). Nevertheless, some spines may not be detected due to their small sizes and poor signal-to-noise ratio in the imaging. Although the breakup of protrusions we described is similar to that in other studies (Harris et al., 1992), we are probably underestimating the number of small spines or stubbies, although this should not affect our conclusions. Ultrastructural reconstructions of the imaged spines (Dunaevsky et al., unpublished findings) will clarify these potential problems.

Finally, visual scoring may introduce a bias in the classification of protrusions, although in this respect we followed the morphological guidelines set by previous structural studies (Harris et al., 1992). Overall, the fact that scoring was consistent in the case of stubbies and spines, where most protrusions were similarly scored during the time-lapse sequences, suggests that the criteria for judging each category were applied consistently. Moreover, since the scoring was done blind to the frame number and the observer did not know which exact protrusion he was scoring, possible subjective influences on the classification were minimized.

Widespread Mixing Among Categories and Special Plasticity of Filopodia

With these caveats, we observed different behavior between filopodia and the rest of the protrusions. Whereas stubbies and spines were likely to be classified in the same category during the time-lapse sequences, filopodia were likely to be scored as a different type of protrusion. The stability of the stubbies and spines that we observed in the midst of rapid spine motility and morphological plasticity confirmed the intuition of previous morphological studies (Jones and Powell, 1969; Peters and Kaiserman-Abramof, 1970; Harris et al., 1992; Fiala et al., 1998), suggesting that these categories represent different types of spines with perhaps different cytoskeletal components that are responsible for their different shapes. At the same time, our study only spanned a few hours, and if the drift among categories was accumulative and the mixing that we detected still did not represent a dynamic equilibrium, it is possible that on a time scale of a few days very few protrusions would conserve their original morphology. On the other hand, since rapid spine motility is developmentally regulated (Dunaevsky et al., 1999), we predict that in mature neurons, less mixing among categories would occur. Longer time-lapse sequences and studies with older neurons could address these issues.

Finally, we observed especially dynamic behavior in filopodia. This confirms the idea that filopodia are an inherently different type of protrusion (Purpura, 1975). We found that filopodia can become stubbies or spines but that they can also originate from stubbies or spines. In other words, rather than detecting a unidirectional transition of their fate, we encountered a feedback loop, which could reflect an unstable state in the fate of the filopodia; this does not agree with a simple model where filopodia are precursors to spines. It therefore becomes important to explore whether the differences in the dynamic behavior of filopodia and other protrusions translate into separate functional roles for these structures.

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REFERENCES

Arnold D, Feng L, Kim J, Heintz N. 1994. A strategy for the analysis of gene expression during neural development. Proc Natl Acad Sci USA 91:9970–9974.
Dailey ME, Smith SJ. 1996. The dynamics of dendritic structure in developing hippocampal slices. J Neurosci 16:2983–2994.
Denk W, Strickler JH, Webb WW. 1990. Two-photon laser scanning fluorescence microscopy. Science 248:73–76.
DeRobertis EDP, Bennett HS. 1955. Some features of the submicroscopic morphology of synapses in frog and earthworm. J Biophys Biochem Cytol 1:47–58.
Dunaevsky A, Tashiro A, Majewska A, Mason CA, Yuste R. 1999. Developmental regulation of spine motility in mammalian CNS. Proc Natl Acad Sci USA 96:13438–13443.
Engert F, Bonhoeffer T. 1999. Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature 399:66–70.
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.
Gray EG. 1959. Electron microscopy of synaptic contacts on dendritic spines of the cerebral cortex. Nature 183:1592–1594.
Harris KM, Kater SB. 1994. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. Annu Rev Neurosci 17:341–371.
Harris KM, Stevens JK. 1989. Dendritic spines of CA1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. J Neurosci 9:2982–2997.
Harris KM, Jensen FE, Tsao B. 1992. Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12:2685–2705.
Heckchen F, Fee M, Tank DW, Denk W. 1999. An in vivo portable two proton microscore. Soc Neurosci Meeting.
Jones EG, Powell TPS. 1969. Morphological variation in the dendritic spines of the neocortex. J Cell Sci 5:509–529.
Kaech S, Brinkhas H, Matus A. 1999. Volatile anesthetics block actin-based motility in dendritic spines. Proc Natl Acad Sci USA 96:10433–10437.
Korkotian E, Segal M. 1998. Fast confocal imaging of calcium released from stores in dendritic spines. Eur J Neurosci 10:2076–2084.
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.
Lo DC, McAllister AK, Katz LC. 1994. Neuronal transfection in brain slices using particle-mediated gene transfer. Neuron 13:1263–1268.
Maletic-Savatic M, Malinow R, Svoboda K. 1999. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. Science 283:1923–1927.
Majewska A, Brown E, Ross J, Yuste R. 2000. Mechanisms of calcium decay kinetics in hippocampal spines: role of calcium pumps and cal-

cium diffusion through the spine neck in biochemical compartmentalization. J Neurosci 20:1722–1734.
Majewska A, Tashiro A, Yuste R. 2000. Spine motility alters calcium decay kinetics due to changes in diffusional coupling across the spine neck. Soc Neurosci Abstract.
Majewska A, Yiu G, Yuste R. In press. A custom made two-proton microscope and deconvolution system. Flügers Archive.
Miller M, Peters A. 1981. Maturation of rat visual cortex. II. A combined Golgi-electron microscope study of pyramidal neurons. J Comp Neurol 203:555–573.
Palay SL. 1956. Synapses in the central nervous system. J Biophys Biochem Cytol 2:193–201.
Papa M, Bundman MC, Greenberger V, Segal M. 1995. Morphological analysis of dendritic spine development in primary cultures of hippocampal neurons. J Neurosci 15:1–11.
Peters A, Kaiserman-Abramof IR. 1970. The small pyramidal neuron of the rat cerebral cortex. The perykarion, dendrites and spines. J Anat 127:321–356.
Purpura DP. 1975. Dendritic differentiation in human cerebral cortex: Normal and aberrant development patterns. In: Kreutzberg GW, editor. Advances in neurology. New York: Raven Press. p 91–116.
Ramón y Cajal S. 1888. Estructura de los centros nerviosos de las aves. Rev Trim Histol Norm Pat 1:1–10.
Ramón y Cajal S. 1891a. Significación fisiológica de las expansiones protoplásmicas y nerviosas de la sustancia gris. Rev Cie Med Barcelona 22:23.
Ramón y Cajal S. 1891b. Sur la structure de l'ecorce cerebrale de quelques mamiferes. Cellule 7.
Ramón y Cajal S. 1893. Neue Darstellung vom Histologischen Bau des Centralnervensystem,. Arch Anat Entwickl 319–428.
Ramón y Cajal S. 1894. La fine structure des centres nerveux. The Croonian lecture. Proc R Soc Lond [Biol] 55:443–468.
Ramón y Cajal S. 1904. La textura del sistema nerviosa del hombre y los vertebrados. Madrid: Moya.
Shepherd G. 1996. The dendritic spine: a multifunctional integrative unit. J Neurophysiol 75:2197–2210.
Yuste R, Denk W. 1995. Dendritic spines as basic units of synaptic integration. Nature 375:682–684.
Yuste R, Holthoff K, Majewska A. 2000. From form to function: calcium compartmentalization in dendritic spines. Nat Neurosci (in press).
Ziv N, Smith S. 1996. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17:91–102.