Dendritic Spines of Developing Rat Cortical Neurons in Culture

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Abstract

The formation of spines and their association with synapses were examined in developing cultured rat cortical neurons using fluorescence labeling techniques. Small protrusions were found on the processes of cultured cortical neurons after seven days *in vitro* (DIV), and the density of protrusions almost halved during the second week *in vitro*, after which it remained unchanged throughout the third week *in vitro*. The proportion of protrusions associated with the accumulation of the presynaptic marker, synaptophysin, increased steadily from <5% at 7 DIV to ~50% at 21 DIV. Based on the absence or presence of an enlargement at the end, protrusions on processes were further divided into filopodia and spines, respectively. The percentage of protrusions that were classified as spines increased steadily from ~5% at 3-4 DIV to ~80% at 18-20 DIV. The percentage of spines associated with synaptophysin accumulation increased gradually as the cortical neurons developed *in vitro*, reaching a plateau of ~40% after two weeks. However, the percentage of filopodia associated with synaptophysin accumulation never exceeded 5% during the first three weeks *in vitro*. Double-label staining the microfilaments and β-tubulin or phosphorylated neurofilament H of cultured neurons further revealed many spines without any nearby axon-like processes. These findings suggest that spines are the dominant form of protrusion on the processes of more mature cortical neurons, that spines are the preferential sites where synapses reside, and that maintaining constant contact with axons is not essential for the formation of spines in cultured cortical neurons.

Key Words: dendritic spine, filopodium, synatogenesis

Introduction

Synapses are the cellular specialization through which neurons communicate with each other. In the cortex of mammalian CNS, most excitatory synapses are made on numerous small protrusions extending from the dendrites of neurons, which are called dendritic spines (11, 12). A typical dendritic spine includes an enlarged head region and a narrower neck region that connects the head region to the dendritic shaft. Spines of other shapes have also been reported (8, 13, 20). Most excitatory synapses are located at the heads of spines (8, 22, 23). Synaptic glutamate receptors are enriched in the plasma membrane of spines opposite to the presynaptic active zones. Underneath this glutamate receptor-rich membrane region lies a compact protein structure called postsynaptic density. Smooth endoplasmic reticulum, also called the spine apparatus, and microfilaments are also found in the spines (21). Spines undergo various movements, and the assembly and disassembly of microfilaments and movements driven by some motor proteins are believed to underlie the structural changes of spines (5, 6, 9, 10, 17, 19, 31). Dendritic spines have been proposed to function as integrative units in synaptic transmission and biochemical

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compartmentation for intracellular second messengers (16, 25, 26).

Dendritic spines of hippocampal and cerebellar neurons have been extensively studied, and the formation of dendritic spines in these areas of the brain appears to be regulated by various mechanisms (1, 3, 8, 12, 24, 27, 31). Cerebral cortex is a major structure of mammalian brains. Recent research of hereditary mental retardation syndromes has revealed that Fragile X and Down syndromes are accompanied by changes in spine morphology, and particularly by a decrease in the number of mature spines and an increase in the number of elongated protrusions, called filopodia (14, 15). The process of spine formation and the relationship between the spine formation and synaptogenesis of cerebral cortical neurons are of interest. Fluorescence labeling techniques clearly reveal small protrusions on cultured cortical neurons. This study reports the change in the densities of protrusions on processes, the change in the shapes thereof, and the change in the proportion of protrusions associated with the accumulation of a presynaptic marker, synaptophysin, in cultured cortical neurons during their *in vitro* development. Furthermore, large numbers of dendritic spine-like protrusions that were not associated with synapses or other processes were observed. The implications of these latter observations for the mechanisms that underlie the formation of dendritic spines in cortical neurons were also discussed.

Materials and Methods

Materials

Pregnant Sprague-Dawley rats were obtained from National Animal Laboratory, Taipei, Taiwan. Hepes, glucose, poly-L-lysine, cysteine, Triton X-100, bovine serum albumin, papain and DNaseI were purchased from Sigma (St. Louis, MO, USA). Calcium-, magnesium-free Hanks' balanced salt saline (HBSS) and minimum essential medium (MEM) powder was obtained from Gibco (Frederick, MD, USA). Strychinine and bicuculline were purchased from Research Biochemicals International (Natick, MA, USA). Horse serum and fetal calf serum were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Mouse anti-synaptophysin and mouse anti-β-tubulin antibodies were obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). Monoclonal SMI 31 antibody to phosphorylated neurofilament H (Sternberg Monoclonal In., USA) was a generous gift from Dr. Wei-Jin Lin of Yang Ming University (Taipei, Taiwan). Texas-Red-conjugated goat anti-mouse IgG, Oregon-Green-conjugated phalloidin and Lucifer-Yellow were purchased from Molecular Probes (Eugene, OR, USA). Other reagents were obtained from Merck-Schduardt (Darmstadt, Germany).

Cortical Cell Culture

Neuron-enriched primary cortical cultures were prepared from Sprague-Dawley rats as described elsewhere (4). In brief, cortices were dissected from rat embryos (18th day of gestation) and treated with papain (10 units/ml). Afterwards, the dissociated cells were washed and suspended in MEM supplemented with 5% horse serum and 5% fetal calf serum. Cells, at the density of 2.5×10^4 cells/cm^2, were then plated on microscopic cover-glasses (18 mm in diameter, Assistant, Germany), pre-coated with poly-L-lysine and placed in the wells of 12-well culture plates, and incubated at 37°C in a humidified incubator with 5%CO_2/95% air for 3 days. Thereafter, cells were treated with 5 μM ARC for 1 day. The culture medium was subsequently replaced with 5% horse serum, and 5% fetal calf serum /MEM every 5 days.

Lucifer-Yellow Injection

Cells with a pyramidal appearance (e.g., Fig. 1A) were first recorded by conventional patch-clamp techniques (29) with borosilicate electrodes filled with the internal solution (in mM: K^+-gluconate 140, EGTA 2, Hepes 10, glucose 10, ATP-Na_2 4, Lucifer-Yellow 9, pH 7.4, resistance 5-8 MΩ). Cells were superfused by the external solution (in mM: NaCl 145, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, Hepes 10, Glycine 0.01 at pH 7.4) containing 30 μM strychinine, and 20 μM bicuculline. After checking the membrane resistance, voltage-dependent responses and spontaneous excitatory postsynaptic currents, the external solution in the recording chamber was slowly replaced by PBS containing 4% paraformaldehyde and 4% sucrose. Currents (-1~-5 pA, 0.5 Hz, duration 1.5 s) were injected into the neuron for 30 min through the recording electrode with the amplifier switched to the current-clamp mode to inject Lucifer-Yellow into the neuron. After injection, cells were washed three times with PBS before further experiments.

Fluorescence Immunolabeling

Lucifer-Yellow-filled cells were fixed again in the fixation solution (4% paraformaldehyde, 0.5% glutaraldehyde and 4% sucrose in PBS) at room temperature for 30 min. After washing with PBS for three times, cells were incubated sequentially with fetal calf serum (1%) at 4°C for 1 hr, and finally with mouse anti-synaptophysin antibody (1 μg/ml PBS) at room temperature for 2 hr. The cells were then incubated with Texas-Red-conjugated goat anti-mouse

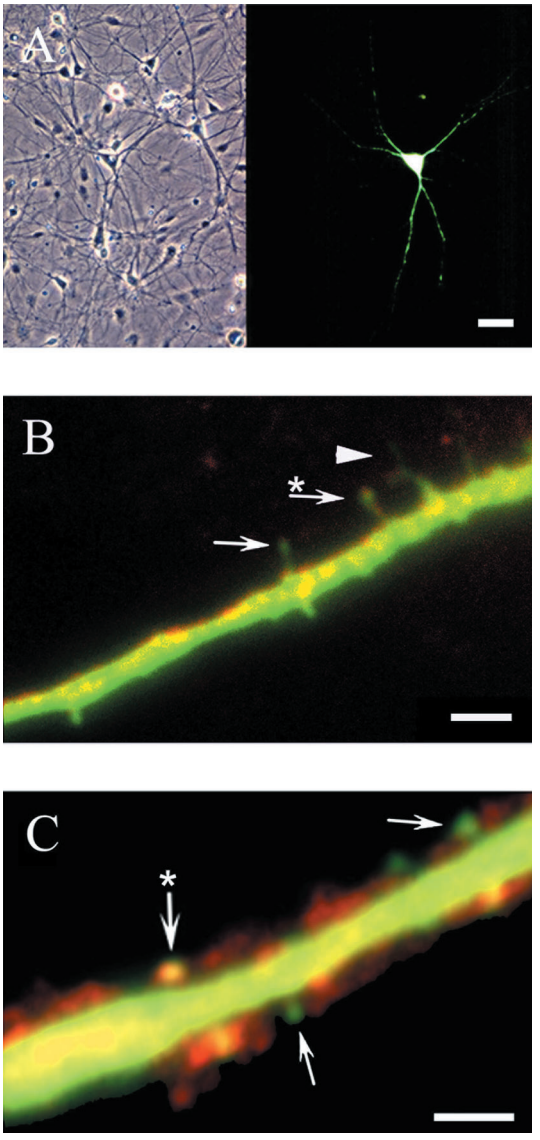

Fig. 1. Fluorescence micrographs of cultured cortical neurons. (A) A Lucifer-Yellow-filled cortical neuron among other cortical neurons at 14 DIV under bright field microscopy (left) and fluorescence microscopy (right). Scale bars: 50 μm. (B and C) Processes of Lucifer-Yellow-filled neurons (green) labeled by the antibody to synaptophysin (red) of a 1- and 3-week-old cortical neuron, respectively. Scale bars in B-C: 2.5 μm. Labels: arrows, spines; arrowheads, filopodia; asterisks, synaptophysin accumulation.

IgG (5 μg/ml) at room temperature for 2 hr. For doubly labeling cells with Oregon-Green-conjugated phalloidin and the antibody to β-tubulin, synaptophysin or phosphorylated neurofilament H, cells were washed with PBS at 37°C and then fixed in the fixation solution at 4°C for 30 min. Cells were then permeabilized by treating with PBS containing 0.05% Triton X-100 at room temperature for 15 min, treated with 1% fetal calf serum at 4°C for 1 hr for blocking non-specific binding, and incubated with Oregon-Green-conjugated phalloidin (50 units/ml) at room temperature for 30 min. After washing three times with PBS, cells were incubated with the primary antibody (mouse anti-synaptophysin, mouse anti-β-tubulin or SMI 31 antibody) at room temperature for 2 hr and subsequently incubated with Texas-Red-conjugated goat anti-mouse IgG at room temperature for 2 hr. After washing three times with PBS, cover slips were mounted with Prolong Antifade (Molecular Probes, Inc.). An epifluoresence microscope (Nikon, Optiphot-2) was used in this study. Photomicrographs were prepared from digital scans of slides with a Nikon slide scanner (Cool Scan III). The contrast and brightness were adjusted digitally by Photoshop software.

Calculation and Statistics

Protrusions with lengths shorter than 6 μm on the processes of pyramidal-shaped neurons were subjected to analyses here. In Lucifer-Yellow-filled neurons, only those protrusions within 150 μm from the somata were included for analyses. On the other hand, in Oregon-Green-conjugated phalloidin-labeled neurons, protrusions on various parts of the processes of pyramidal-shaped neurons were subjected to analyses. The fine protrusions on the processes of Oregon-Green-conjugated phalloidin-labeled neurons were further divided into two groups: filopodia and spines. A filopodium was defined as a protrusion that tapered from the end attaching to a process toward the end pointing away from the process. A spine was defined as a protrusion with the end pointing away from the process being bigger in diameter than the remaining part of this protrusion. Since filopodia and spines could rapidly change their morphology into on another (7), our analyses represented the percentages of filopodia and spines of the total protrusions on processes at the instant when neurons were fixed. Data were analyzed by Student's *t* test or by one-way ANOVA followed by Duncan's multiple range test for multiple comparison.

Results

Pyramidal-shaped neurons were impaled by patch-clamp electrodes that were filled with an internal solution containing Lucifer-Yellow to study the protrusions on the processes of cultured rat cortical neurons. The membrane resistance, voltage-dependent sodium and potassium currents and spontaneous excitatory postsynaptic currents were measured, and then Lucifer-Yellow was injected into the neurons. Figure 1A shows a cultured cortical neuron labeled with Lucifer-Yellow, among many unlabeled neurons, as observed under bright-field and fluorescence microscope. At higher magnification, many protru-

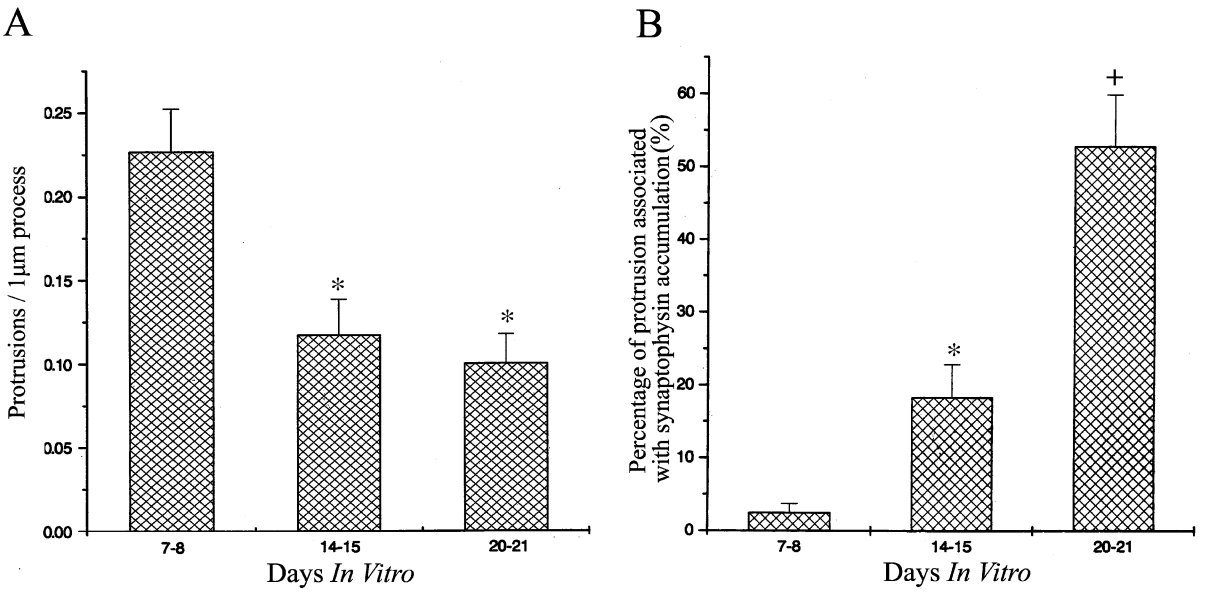

Fig. 2. (A) The densities of the protrusions on the processes of Lucifer-Yellow-filled 1-, 2- and 3-week-old neurons. Data are the means±S.D. (n = 8). *: significantly different from neurons at 7-8 DIV, $P < 0.05$. (B) The percentages of the protrusions that were associated with synaptophysin accumulation on the processes of Lucifer-Yellow-filled 1-, 2- and 3-week old neurons. Data are means±S.D. (n = 8). *: significantly different from neurons at 7-8 DIV, $P < 0.05$. +: significantly different from neurons at 14-15 DIV, $P < 0.05$.

sions were observed on the processes of cultured neurons (Figs. 1B and 1C). The protrusions on the processes of the cultured neurons between 7 and 21 DIVs were subject to quantitative analyses. The gradual weakening of the fluorescence intensity from the somata toward the ends of the processes of Lucifer-Yellow-filled neurons was such that only protrusions within 150 μm of the somata and with lengths of under 6 μm were included in the analyses. The density of protrusions on the processes of neurons at 7-8 DIV significantly exceeded those in neurons at 14-15 and 20-21 DIVs (Fig. 2A), and no significant difference was observed between the densities in neurons at 14-15 and 20-21 DIVs. Immunostaining Lucifer-Yellow-filled neurons with the antibody to synaptophysin, a marker for presynaptic terminals, revealed that some of the protrusions were associated with synapses, as indicated by synaptophysin accumulation (Figs. 1B and 1C), and the percentages of the protrusions associated with synapses increased steadily during the first three weeks *in vitro* (Fig. 2B). A large proportion of synaptophysin puncta was found to be associated with the shafts of processes (Figs. 1B and 1C). This observation was consistent with those made from rat hippocampal neurons (2) and retinal ganglion cells (30) in culture.

The protrusions on the processes of cultured cortical neurons had diverse structures (such as those indicated by the arrows and arrowheads in Fig. 1B). Cultured cortical neurons between DIVs 3 to 20 were fixed and labeled with Oregon-Green-conjugated phalloidin, which specifically binds F-actin that is greatly enriched in dendritic spines, to analyze the changes in the structure of the protrusions on the processes of cultured rat cortical neurons during their in vitro development (Figs. 3A-3D). Morphological differences enable protrusions to be further divided into spines (labeled by arrows) and filopodia (labeled by arrowheads) based on the presence or absence, respectively, of an enlargement at the end. Starting from 3-5 DIV, the presence of spines and filopodia on the processes of cultured neurons was evident (Figs. 3A-3D). Quantitative analyses indicated that the percentage of dendritic protrusions that were classified as spines increased from ~5% at 3-5DIV to ~80% at 18-20 DIV (Fig. 4).

Whether the gradual change in the structure of protrusions on the processes of the cultured cortical neurons was related to the process of synapse formation was studied. The Oregon-Green-conjugated phalloidin-labeled cells were immunostained with the antibody to synaptophysin. The results showed that most synapses, as indicated by synaptophysin accumulation (asterisks in Fig. 3A), were made on the shafts of processes of the cultured neurons at 3-5 DIV, whereas various proportions of synapses were co-localized with protrusions in neurons beyond 8-10 DIV (Figs. 3B-3D). The percentage of spines that were associated with synapses increased from ~3% at 3-5 DIV to ~40% at 13-15 and 18-20 DIVs (Fig. 5). In

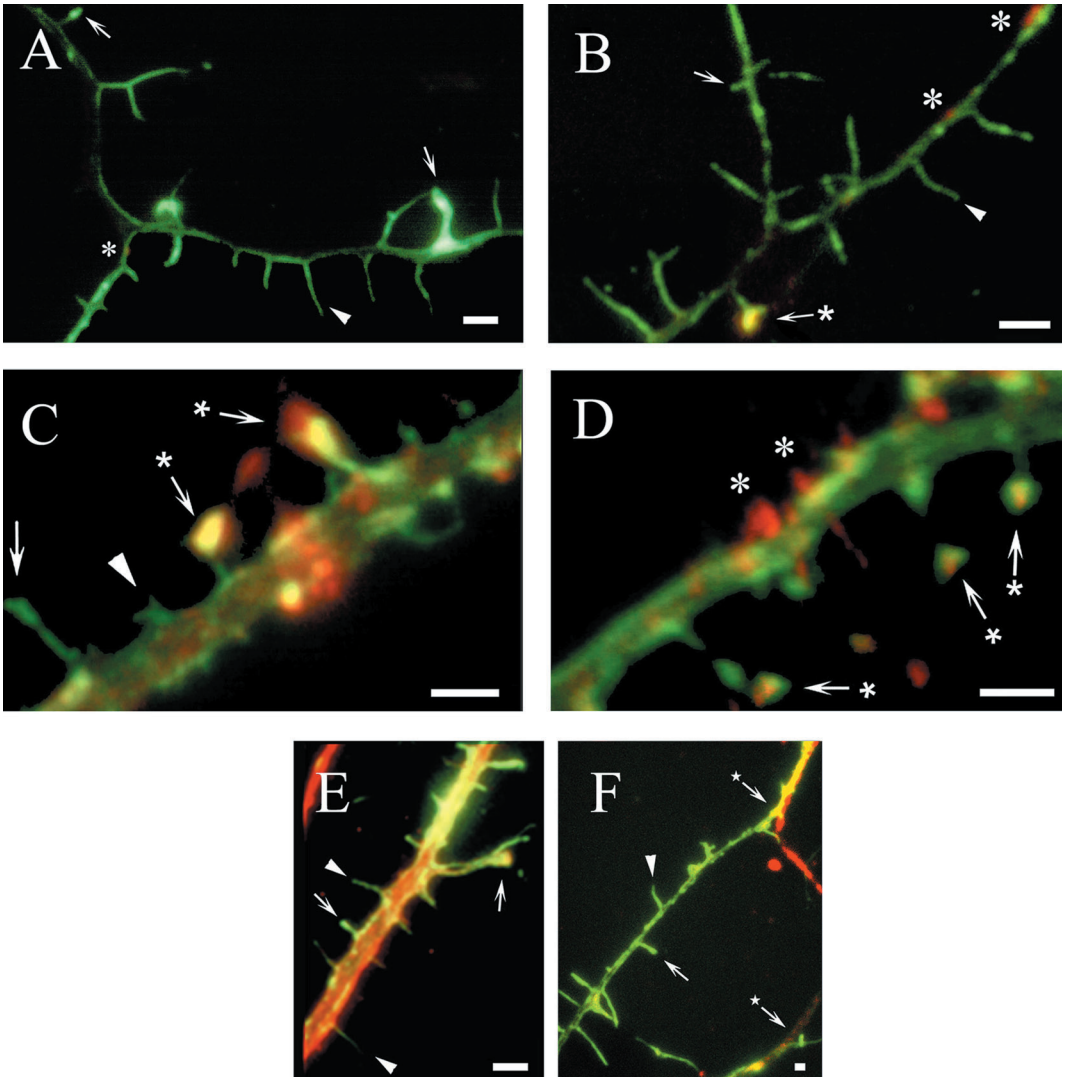

Fig. 3. (A, B, C, D) Processes of cultured cortical neurons at 4, 10, 14 and 18 DIV, respectively, labeled by Oregon-Green-conjugated phalloidin (green) and the antibody to synaptophysin (red). (E) Processes of cultured cortical neurons at 14 DIV labeled with Oregon-Green-conjugated phalloidin (green) and the antibody to β-tubulin (red). (F) Processes of cultured cortical neurons at 14 DIV labeled with Oregon-Green-conjugated phalloidin (green) and the antibody SMI 31 to phosphorylated neurofilament H (red). Scale bars in A-E: 2.5 μm. Scale bar in F: 1 μm. Labels: arrows, spines; arrowheads, filopodia; asterisks, synaptophysin accumulation; stars, spines associated with axons.

contrast, the percentage of filopodia that were associated with synapses never exceeded 5% during the first three weeks *in vitro* (Fig. 5). The results indicated that synapses preferentially reside on spines rather than on filopodia. However, the data also showed that under 50% of the spines in 1-, 2- and 3-week-old cortical neurons were associated with synapses. In Figs. 1B and 1C, spines not associated with synaptophysin accumulation were labeled by arrows alone.

The synaptophysin-staining method used herein may have labeled only the more mature presynaptic terminals, which contained more synaptic vesicles, but not the immature ones, which might contain only few vesicles. To examine this possibility, cultured cortical neurons were labeled with Oregon-Green-conjugated phalloidin and immunostained with the antibody to β-tubulin to reveal both dendrites and axons in the vicinity of spines. Many spines without any nearby processes, except for those from which they originated, were observed (Figure 3E shows an example). Another possibility was that some protrusions identified herein as spines or filopodia without any nearby processes were actually of axonal origin and not parts of dendrites. To examine this possibility, Oregon-Green-conjugated phalloidin-labeled neurons were immunostained with the antibody to phosphorylated neurofilament H (SMI 31), which interacts strongly with axons (28). In these doubly labeled

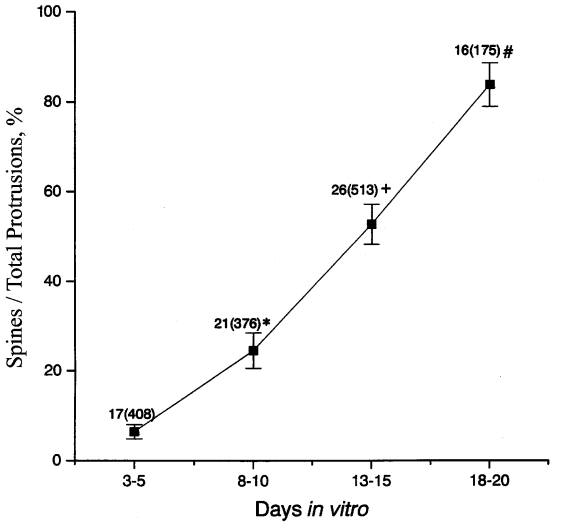

Fig. 4. Percentage of spines on the processes of Oregon-Green phalloidin-lableled cultured cortical neurons at different times in vitro. Data are means±S.D. The number and the number in parenthesis on top of each point are respectively the number of cells and the number of total protrusions used for analyses. *: significantly different from neurons at 3-5 DIV, $P < 0.05$. +: significantly different from neurons at 8-10 DIV, $P < 0.05$. #: significantly different from neurons at 13-15 DIV, $P < 0.05$.

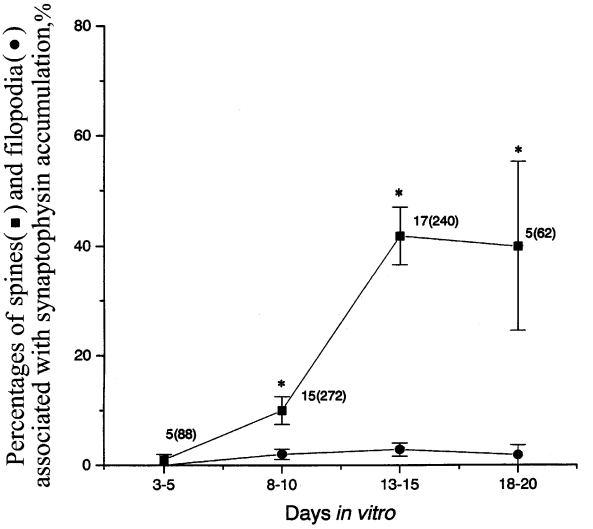

Fig. 5. Percentages of spines and filopodia associated with synaptophysin accumulation on the processes of cultured cortical neurons at different times in vitro. Data are means±S.D.. The number and the number in parenthesis on top of each data point are respectively the number of cells and the number of protrusions (including both spines and filopodia) used for analysis. *: significantly different from the percentage of filopodia associated with synaptophysin accumulation of neurons at the same time *in vitro*, $P < 0.05$.

neurons, two kinds of processes could be found. One kind of processes was heavily stained with SMI 31 and sparsely labeled with phalloidin, and the other was heavily labeled with phalloidin and only sparsely stained by SMI 31 (Fig. 3F). These two kinds of processes were likely to represent axons and dendrites of cultured cortical neurons, respectively. Figure 3E shows spines originating from dendrites and without any nearby SMI 31-positive processes, as well as spines that originated from dendrites and formed contacts with SMI 31-positive processes. These observations indicated the presence of dendritic spines without forming any contact with axons in cultured rat cortical neurons.

Discussion

This study reports that the density of the protrusions on the processes of cultured rat cortical neurons significantly decreases between 7 and 14 DIVs, but does not undergo further significant changes between 14 and 21 DIVs. The proportion of protrusions on neuronal processes that are classified as spines increases steadily from ~5% at 3-5 DIV to over 80% at 18-20 DIV, whereas the proportion of the protrusions classified as filopodia changes oppositely during the same period. These results indicate that filopodia and spines represent the dominant protrusions made on the processes of immature and more mature cultured cortical neurons, respectively.

The observation that spine-like protrusions associated with synapses greatly outnumber filopodium-like protrusions associated with synapses further suggests that spines, rather than filopodia, are the preferred sites for formation of synapses in cultured rat cortical neurons. The percentage of spines associated with synapses in cultured cortical neurons increases gradually in the first two weeks *in vitro*, reaching a plateau during the third week *in vitro*. This temporal profile is in parallel with those of the functional properties of excitatory synapses of these neurons during the same period. Our earlier electrophysiological studies indicated that the frequencies and amplitudes of spontaneous excitatory postsynaptic currents of cultured cortical neurons increased significantly during the second week *in vitro*, remaining virtually unchanged during the third week *in vitro* (18). Together, these anatomical and functional studies reveal that the excitatory synapses in cultured cortical neurons are made and mature mostly during the first two weeks *in vitro*, before entering a steady state during the third week *in vitro*. Surprisingly, this study further indicates that during this latter period, while excitatory synapses are in a steady state, fewer than half of the spines on the processes of the cultured cortical neurons are associated with synapses or axons.

A hypothesis has been proposed that filopodia are a subcellular specialization that rapidly extends and retracts from dendrites, seeking potential synapse-formation partners near the dendrites and becoming transformed into more stable spine-like structures after synapses are formed on them (5, 8, 19, 31). According to this hypothesis, the formation of spines on dendrites is closely related to synaptogenesis. However, studies have revealed that the cerebellar Purkinje cells of reeler and weaver mice still form spines even in the absence of appropriate excitatory inputs (1, 3, 27), suggesting that spine formation in cerebellar Purkinje cells is regulated by an intrinsic program and is independent of synaptogenesis. The observations herein of large numbers of spines not associated with synapses or forming contacts with axons show that, as for the cerebellar Purkinje cells, synapse formation is unlikely to dominate the formation of spines on the dendrites of cultured rat cortical neurons.

However, the possibility that spines are indeed transformed from filopodia and that this transformation involves only “transient”, but not “long-lasting” contacts between filopodia and axons, cannot be excluded. It still remains possible that the spines associated and unassociated with synapses may represent two sub-populations of spines, like the spines and protospines described by Fiala *et al*. (1998) in hippocampal neurons. Further studies that use time-lapse recording of dendritic protrusions in developing cultured cortical neurons should be performed to examine these possibilities.

Acknowledgments

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