

Activity-Dependent PSD Formation and Stabilization of Newly Formed Spines in Hippocampal Slice Cultures

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Development and remodeling of synaptic networks occurs through a continuous turnover of dendritic spines. However, the mechanisms that regulate the formation and stabilization of newly formed spines remain poorly understood. Here, we applied repetitive confocal imaging to hippocampal slice cultures to address these issues. We find that, although the turnover rate of protrusions progressively decreased during development, the process of stabilization of new spines remained comparable both in terms of time course and low level of efficacy. Irrespective of the developmental stage, most new protrusions were quickly eliminated, in particular filopodia, which only occasionally lead to the formation of stable dendritic spines. We also found that the stabilization of new protrusions was determined within a critical period of 24 h and that this coincided with an enlargement of the spine head and the expression of tagged PSD-95. Blockade of postsynaptic AMPA and NMDA receptors significantly reduced the capacity of new spines to express tagged PSD-95 and decreased their probability to be stabilized. These results suggest a model in which synaptic development is associated with an extensive, nonspecific growth of protrusions followed by stabilization of a few of them through a mechanism that involves activity-driven formation of a postsynaptic density.

Keywords: confocal imaging, dendritic spine, hippocampus, plasticity, postsynaptic density, synaptogenesis

Introduction

Neuronal activity modulates excitatory synaptic properties and function in many different ways. Inhibition or enhancement of firing or synaptic activity results in an up scaling or down scaling of glutamate sensitivity, an effect that appears to be mainly mediated by changes in receptor expression mechanisms (Turrigiano et al. 1998; Turrigiano and Nelson 2004). Also patterns of synaptic activation, that induce properties of synaptic plasticity, such as long-term potentiation or depression, result in modifications of receptor cycling and expression at the synapse (Nicoll 2003; Kennedy and Ehlers 2006; Nicoll et al. 2006). However, in addition to these homeostatic and activity-dependent regulations of receptor expression, several recent studies have provided evidence for mechanisms of activity-dependent synaptogenesis and synapse remodeling. Confocal analysis of dendritic spines, which are the sites of most excitatory synapses in the brain, showed that they are not as stable structures as previously thought (Yuste and Bonhoeffer 2004; Segal 2005). In vitro experiments showed that they can be formed de novo within short periods of time as a result of synaptic activation or induction of synaptic plasticity (Engert and Bonhoeffer 1999; Maletic-Savatic et al. 1999; Toni et al. 1999). Under in vivo conditions, they undergo a continuous turnover and replacement that are developmentally regulated, appear to

be region specific, and modulated by sensory activity (Grutzendler et al. 2002; Trachtenberg et al. 2002; Zuo, Lin, et al. 2005; Zuo, Yang, et al. 2005; Holtmaat et al. 2006). These results have thus strengthened the possibility that learning processes not only involve synapse-specific modifications of synaptic strength but may also be associated with a remodeling of synaptic connections through competitive mechanisms of synapse or spine formation, stabilization, or elimination (Stepanyants et al. 2002). These processes, however, remain poorly understood (Garner et al. 2006). It remains unclear how exactly are new spines formed, what is the efficacy of the process, how fast are new, stable spines generated, what regulates the stabilization of a new protrusion? To address these issues, we developed here a time-lapse confocal analysis applied to hippocampal organotypic slice cultures. We find that turnover in this developmental system affects a high proportion of protrusions over 24 h and shares many similarities with what has been reported in vivo, including its developmental regulation. Furthermore, the accessibility of the system made it possible to analyze important properties of newly formed protrusions. We find that most new protrusions are only transient and only occasionally lead to the formation of stable spines and this with markedly different efficacies depending upon protrusion type. We find also that the stabilization of a new protrusion occurs over a critical period of 24 h, that this process is associated with an increase in the size of the spine head and correlates with activity-driven mechanisms of postsynaptic density (PSD) expression.

Materials and Methods

Cultures

Transverse hippocampal organotypic slice cultures (400 μ m thick) were prepared from 6- to 7-day-old rats using a protocol approved by the Geneva Veterinarian Office (authorization 31.1.1007/3129/0) and maintained for 11–29 days in culture as described (Stoppini et al. 1991). Slice cultures of 2 different ages (11 and 25 days in vitro [DIV]) were specifically analyzed in this study because these periods correspond to different phases of synaptic development in slice cultures (Buchs et al. 1993; Collin et al. 1997). In order to facilitate transfer of slice cultures to recording conditions, they were cultured on a small membrane confetti (6–8 mm in diameter, Millipore, Billerica, MA, USA) placed on top of a Millipore insert. In all experiments, slice cultures were maintained in a CO₂ incubator at 33 °C. For the visualization of spines, slice cultures were transfected with a pcDNA3-EGFP (enhanced green fluorescent protein) plasmid using a biolistic method (Helios Gene Gun, Bio-Rad, Hercules, CA, USA) 3 days before the first observation. The number of transfected CA1 pyramidal cells varied between 0 and 5 per slice culture; fluorescence usually started to be expressed after 24–48 h and then remained stable for at least 10–15 days. To examine PSD formation in newly formed spines, slice cultures were cotransfected with a pcDNA-EGFP plasmid and a pcDNA-PSD-95-DsRed2 plasmid. Cotransfection was effective in 15 out of 16 cases.

Confocal Imaging and Analysis

Laser scanning microscopy was realized with an Olympus Fluoview 300 system using a 488-nm Argon laser or a 2-photon laser set at 920 nm (Coherent, Santa Clara, CA, USA). Transfected slice cultures were maintained in a recording chamber under immersion conditions with culture serum for the time of observation (10 min, 25 °C) and then transferred back to the incubator. In each experiment, a complete image of the slice was initially obtained with a 5× objective to allow precise recognition and localization of the dendritic segment under analysis (one segment per slice culture). Also an image of the entire CA1 pyramidal neuron with steps of 3 μm between scans was realized each day using the 40× objective to check for the general morphology and health of the selected neuron. All analyses were carried out on dendritic segments of about 35 μm in length and located between 150 and 300 μm from the soma and imaged with a 40× objective using a 10× additional zoom (final definition: 25 pixels per micron; steps between scans: 0.25–0.5 μm). Control experiments showed that this procedure could be applied up to 10 consecutive times without deleterious effects on cell viability, as indicated by absence of cell death, dendritic beadings, or propidium iodide staining.

PSD-95-DsRed2 was imaged in all experiments using a spinning-disk confocal system (Visitron Systems, Puchheim, Germany) and an excitation laser set at 568 nm, and imaging was only carried out at the end of the experiment to avoid possibilities of phototoxicity. Images were obtained with a 40× objective using z-steps of 0.4 microns with Metamorph software. Maximal intensity projections of the z-stacks were used to analyze the presence or absence of PSD-95-DsRed2 staining. The average intensity of the spine head area (determined in the EGFP image) was measured in the red channel (PSD-95-DsRed2). Positive spines were in average 17% above the threshold level. Spines were considered positive for PSD-95

staining if this average intensity was more than 5% higher than the background determined in an adjacent area. The minimal average intensity for a positive spine was 11% and the maximal 30%.

All analyses were carried out blind by at least 2 independent observers on z-stacks of raw images using a plugin specifically developed for OsiriX software (<http://homepage.mac.com/rossetantoinne/osirix/>). Protrusions were classified as filopodia (protrusions without enlargement at the tip), stubby spines (short protrusions without neck and in direct continuity with the dendritic surface), and mushroom spines (protrusions with a neck and an enlargement at the tip). Determination of spine behavior over time was done by systematic analysis of individual z-stacked images. Also quantifications of spines width were carried out by measuring the largest diameter of the spine head observed on any of the z-stacked confocal images. All statistics are given with the standard error of the mean. Standard *t*-tests were performed to compare Gaussian distributions, and Mann-Whitney tests were used for non-Gaussian distributions. For all tests, α was set to 5%.

Results

Spine Turnover Rate in Hippocampal Slice Cultures

To visualize dendritic spines, CA1 pyramidal neurons in slice cultures were transfected with EGFP at either 8 or 22 DIV and imaged repetitively over the next 3–7 days using confocal microscopy. Control experiments showed that repetitive imaging of the same cells and/or dendritic segments for up to 10 times under the conditions used resulted in no obvious signs of toxicity such as beadings or cell death and no staining

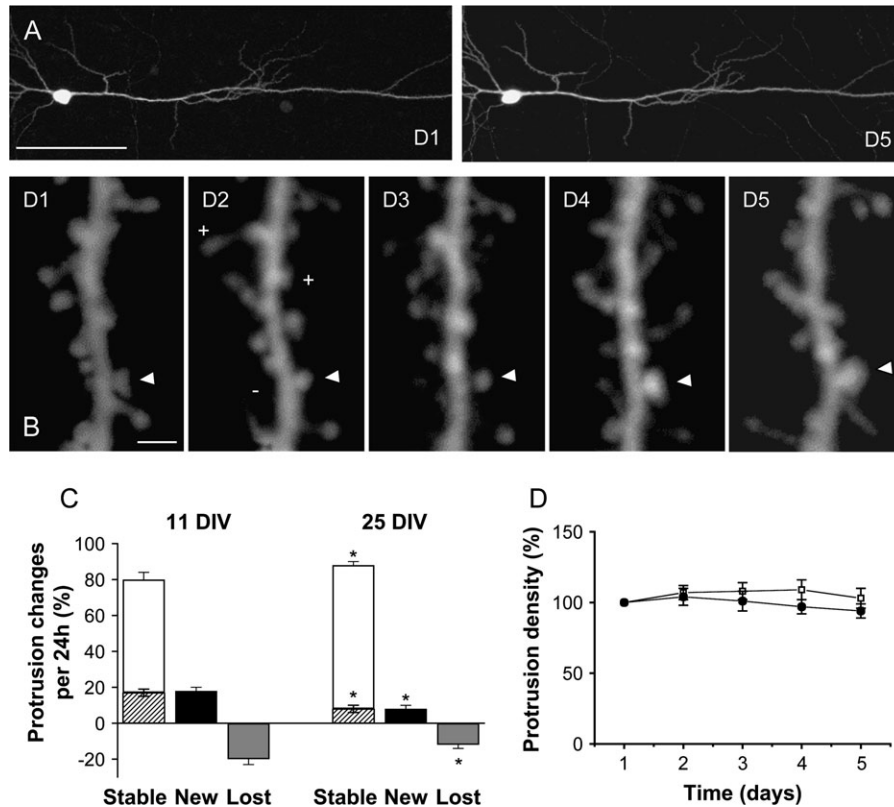


Figure 1. Developmental regulation of protrusion turnover in hippocampal slice cultures. (A) Illustration of a typical CA1 pyramidal neuron imaged 3 (D1) and 8 (D5) days after transfection in an 11 DIV hippocampal organotypic culture. (B) Illustration of protrusion turnover obtained by daily confocal imaging of the same dendritic segment. The arrowhead indicates one stable spine; plus and minus signs are examples of new and lost spines, respectively. (C) Bar graph showing the percent of unchanged, stable spines (white bars), stable spines with changes in morphology (dashed bars), newly formed spines (black bars), and lost spines (gray bars) detected over a 24-h period in 11 DIV and 25 DIV hippocampal cultures. Data are mean \pm standard error of the mean of analyses made on 10 dendritic segments from 10 pyramidal cells per group; $P < 0.05$. (D) Changes in protrusion density expressed in each experiment as percents of initial values in 11 (open squares) and 25 (black circles) DIV cultures ($n = 10$; $P < 0.05$; scale bars: A 100 μm; B 1 μm).

for propidium iodide. Figure 1A illustrates one CA1 pyramidal neuron imaged on days 1 and 5, following a daily analysis of the spine changes occurring on one of its dendritic segments (Fig. 1B). As apparent in this example, numerous changes could be detected over a 24-h period, including transformation or modifications of the shape of spines, appearance of filopodia, formation of new spines, and disappearance of preexisting protrusions. Analyses were carried out on 20 different cells from either 11 or 25 DIV hippocampal slice cultures with daily observations made for 5 consecutive days on a total of 978 protrusions. The data, summarized in Figure 1C, indicate that the changes detected over a 24-h period were considerably larger in all aspects examined in 11 as compared with 25 DIV slice cultures, thus clearly pointing to a developmental regulation of spine turnover and plasticity in slice cultures. The proportion of new protrusions averaged $19 \pm 2\%$ in 11 DIV slice cultures, but only $8 \pm 2\%$ in 25 DIV tissue ($n = 10$ per group; $P < 0.05$), whereas the proportion of lost protrusions decreased from $19 \pm 3\%$ to $12 \pm 2\%$ ($n = 10$; $P < 0.05$). Accordingly, the proportion of stable protrusions increased from $81 \pm 3\%$ to $88 \pm 2\%$ ($n = 10$; $P < 0.05$). Overall, the turnover rate calculated as half of the sum of the ratios of new and lost protrusions observed over 24 h decreased from $19 \pm 2\%$ to $10 \pm 1\%$ between 11 and 25 DIV ($n = 10$; $P < 0.05$).

Interestingly, in addition to new and lost protrusions, we also observed a significant proportion of transformations or changes in spine shape. These included not only transformations of filopodia into stubby or mushroom spines but also transforma-

tions of stubby into mushroom spines (Fig. 3). The proportion of these shape changes also considerably decreased with developmental maturation of slice cultures between DIV 11 and 25 (from $16 \pm 2\%$ to $8 \pm 2\%$; $n = 10$; $P < 0.05$). Finally, as indicated in Figure 1D, all these modifications occurred without detectable changes in protrusion density that remained constant both during the periods of observation (Fig. 1D) and by comparison of 11 and 25 DIV slice cultures (1.02 ± 0.11 vs. 1.06 ± 0.11 protrusions/ $1 \mu\text{m}$, respectively; $n = 10$). All together, these data indicated that dendritic spines in hippocampal slice cultures exhibit a high degree of turnover that is developmentally regulated.

Characteristics of Protrusion Changes

In order to more precisely define the characteristics of new protrusions, we classified them into 3 main categories: filopodia, defined as long protrusions without head or widening at the tip, stubby spines, defined as protrusions without neck, and mushroom spines, protrusions with a neck and a widening at the tip (Fig. 2A). Overall, the proportion between these different protrusions did not vary greatly with development, as the ratios were comparable in 11 and 25 DIV slice cultures, although, as shown in Figure 2B, the proportion of filopodia was slightly but not significantly smaller at 25 DIV ($4 \pm 2\%$ vs. $8 \pm 3\%$). To verify this further and test the accuracy of the confocal analyses, we also carried out an electron microscopic determination of the proportion of these different protrusions. For this,

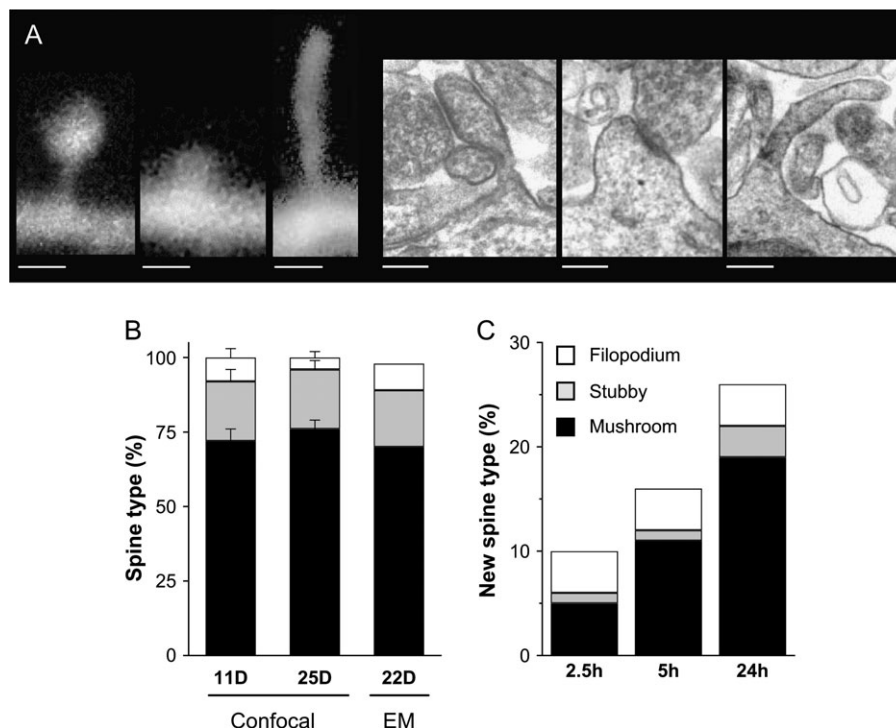


Figure 2. Categorization of spine types in hippocampal cultures. (A) Typical examples of a mushroom spine (left), a stubby spine (center), and a filopodium (right) imaged with confocal microscopy (left panel) and electron microscopy (right panel). (B) Proportion of protrusion types (mushroom spines: black columns; stubby spines: gray columns; filopodia: white columns) detected on dendritic segments imaged with confocal microscopy in 11 and 25 DIV cultures ($n = 10$ cells; 720 protrusions analyzed) and with electron microscopy in 22 DIV cultures ($n = 3$ cultures; 99 protrusions analyzed). (C) Proportion of the different types of new protrusions (mushroom spines: black columns; stubby spines: gray columns; filopodia: white columns) detected using different observation intervals in 11 DIV cultures. Data were obtained through analysis of 9 different cells (25–66 new protrusions analyzed). Note the nonlinearity of results indicating low stability of new protrusions and the high rates of formation of both new spines and filopodia with the shortest interval (scale bar: A $0.5 \mu\text{m}$).

dendritic segments from 3 hippocampal slice cultures of 22 DIV were analyzed from serial sections (Fig. 2*A*, right panel) and the proportion of filopodia, stubby, and mushroom spines determined. As illustrated in Figure 2*B*, the ratios so obtained nicely correlated with the data obtained by confocal microscopy.

Next, we used these categories to analyze the mechanisms through which new spines were generated. For this, we classified all new protrusions detected over different periods of time. As shown on Figure 2*C*, the number of new protrusions

detected by repetitive imaging showed some variability, but clearly depended upon the period of observation, indicating that some protrusions were likely to have a rather short lifetime. For this reason, we also carried out analyses with a short time interval (2.5 h). These data indicated that new protrusions mainly appeared as new mushroom spines (50%) or as filopodia (40%) and that the rates of formation for both types of protrusions were in the range of 2% of existing protrusions per hour, which is rather high, but consistent with previous

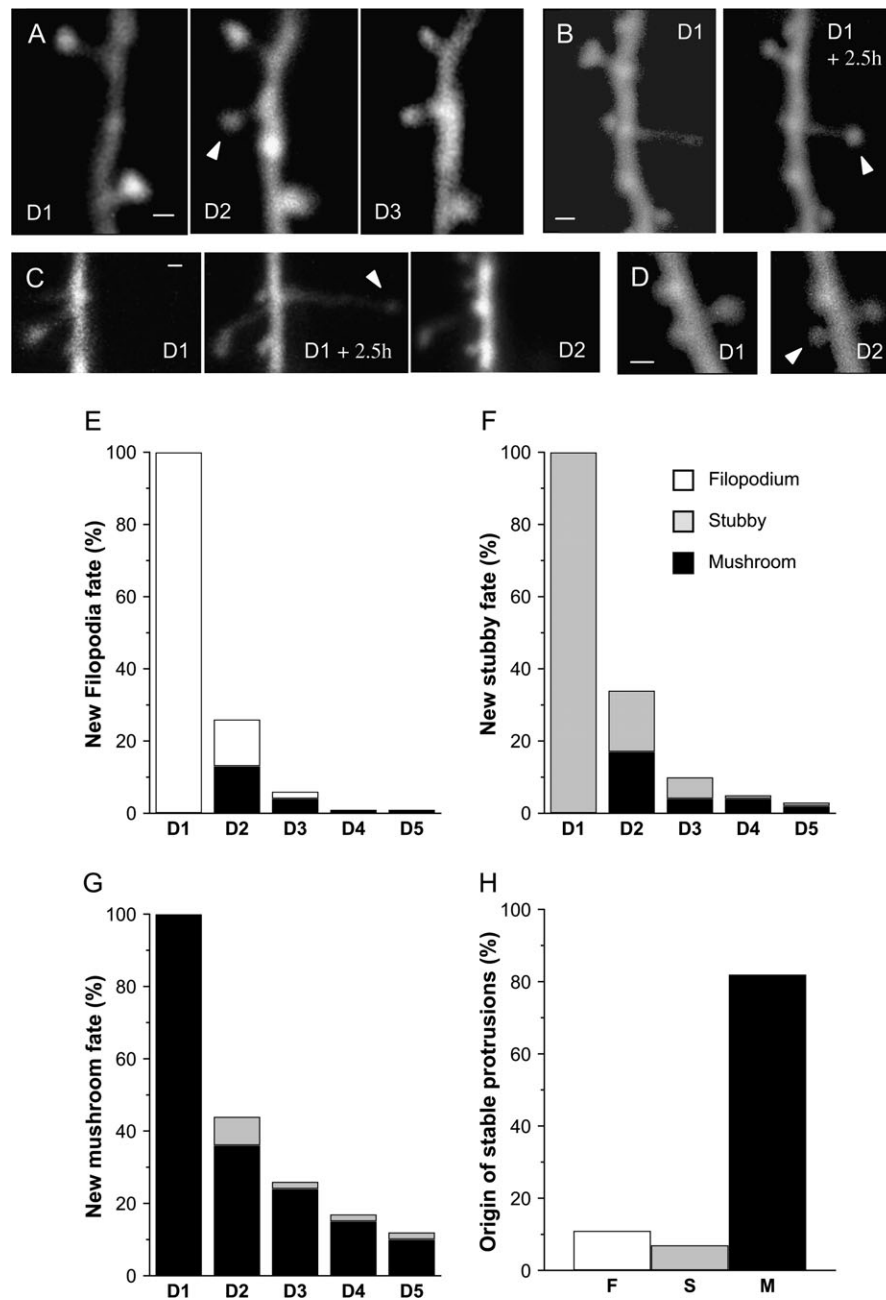


Figure 3. Fate and stability of new protrusions. (*A–D*) Illustration of new protrusions fate. (*A*) New spine that remained stable for >24 h (arrowhead). (*B*) Filopodium that transformed into a mushroom spine (arrowhead). (*C*) Transient filopodium. (*D*) Stubby spine that transformed into a mushroom spine. (*E*) Quantitative analysis of the fate of a pool of 33 new filopodia analyzed in 7 experiments. Data are expressed as percent of the number of initial filopodia still present as filopodia (open bars) or mushroom spines (black bars) on the next 4 consecutive days. (*F*) Same, but for a pool of 29 new stubby spines (gray bars) and their transformations in mushroom spines (black bars). (*G*) Same, but for a pool of 59 new mushroom spines (black bars), among which some could transform back into stubby spines (gray bars). (*H*) Proportion of protrusion types (F: filopodia; S: stubby spines; M: mushroom spines) resulting in the formation of stable dendritic spines exhibiting a minimum 48 h stability in 11 DIV cultures. Note the low efficiency of filopodia in generating stable spines (scale bar: 0.5 μ m).

results obtained through continuous imaging over shorter periods of time (Engert and Bonhoeffer 1999; Jourdain et al. 2003; Nagerl et al. 2004).

Fate and Stability of New Protrusions

In order to analyze the behavior, half-life, and efficacy with which these different new protrusions generated stable synaptic contacts, we next followed the new events detected after the first 2.5 h over 5 consecutive days. A total of 121 new protrusions (33 filopodia, 29 stubby spines, and 59 new mushroom spines) were analyzed together with their transformations. Figure 3*A-D* illustrates some examples of these, namely, a new mushroom spine that remained stable for 2 days (Fig. 3*A*), a new filopodium that transformed into a mushroom spine (Fig. 3*B*), a new filopodium that disappeared on the next day (Fig. 3*C*), and a stubby spine that transformed into a mushroom spine (Fig. 3*D*). These changes were then analyzed quantitatively and summarized in Figure 3*E-G*. As illustrated in Figure 3*E*, new filopodia were very labile structures as 33% of them had already disappeared after 2.5 h (data not shown) and only 13% were still present as filopodia after 24 h, 2% after 48 h, and none after 72 h. Considering an exponential rate of disappearance, one can calculate that the half-life of filopodia was approximately 6 h. Although, in most cases (64%), filopodia simply disappeared within 24 h, in 13% of cases, they transformed into mushroom spines (Fig. 3*E*, black columns). These transformations, however, were usually unstable as well, as most of them (94%) also disappeared within the next 2 days. Overall, only 1% of all filopodia resulted in the formation of mushroom spines stable for over 2 days.

As indicated in Figure 3*F*, stubby spines were also rather unstable protrusions, with only 17% and 9% of them being still present after 1 and 2 days, respectively. This suggests a half-life of about 15 h. A significant proportion of stubby spines (17%), comparable to that of filopodia, was also transformed over 24 h into mushroom spines. However, the stability of these mushroom spines was not better than for filopodia as only 23% of them persisted for the next 24 h. Overall, only 4% of all new stubby spines resulted in the formation of mushroom spines stable for at least 2 days.

Curiously, new mushroom spines were also markedly unstable. Most of them (56%) also disappeared within 24 h, whereas 36% were still present and 8% transformed into stubby spines. Their half-life calculated from their rate of disappearance (Fig. 3*G*) was approximately 20 h. Overall, only 24% of all new mushroom spines persisted for at least 2 days, indicating an important rate of elimination. On a quantitative basis, these results indicate that only 16% of all newly grown protrusions detected over a 2.5-h period resulted in the formation of dendritic spines stable for at least 2 days in 11 DIV slice cultures. Interestingly, this very high initial instability was not related to the developmental stage of the cultures. Similar experiments carried out in 25 DIV slice cultures showed that, although the turnover rate and rate of protrusion formation decreased by a factor of 2 (Fig. 1*C*), the proportion of new protrusions that succeeded in becoming stable remained very low, with about 90% of them being still eliminated within the first 48 h ($n = 10$). Furthermore, considering the relative proportion of the different types of protrusions, it appears that filopodia, although quite numerous, contributed to only 11% of all new mushroom spines exhibiting a minimum 48 h

stability; stubby spines contributed to 7%, whereas 82% of all new stable spines were formed directly as new mushroom spines (Fig. 3*H*).

Stability of Preexisting Protrusions

We next also assessed the stability of preexisting spines. For this, we considered a population of 294 mushroom spines observed on 2 consecutive days and thus exhibiting at least a 24-h period of stability. These spines were then followed by repetitive imaging over the next 4 days and their changes analyzed as shown in Figure 4*A*. Under these conditions, only a small percentage of these preexisting spines disappeared over 24 h (18%), 7% were transformed into stubby spines, and most of them (75%) remained stable. Their half-life calculated from their rate of disappearance was about 90 h or 4 days, thus 4–5 times longer than that of new mushroom spines, indicating the existence of a process of stabilization of newly formed mushroom spines. Similarly, analyses carried out in 25 DIV slice cultures showed that the stability of preexisting spines was still higher and increased with development (Fig. 4*B*) as only 9% of them disappeared over 24 h, and their calculated half-life was then almost 200 h or about 8 days.

To better understand how these changes in stability occurred over time, we next analyzed the stability of newly formed mushroom spines calculated over periods of 24 h and determined at different times after their appearance. For this, we

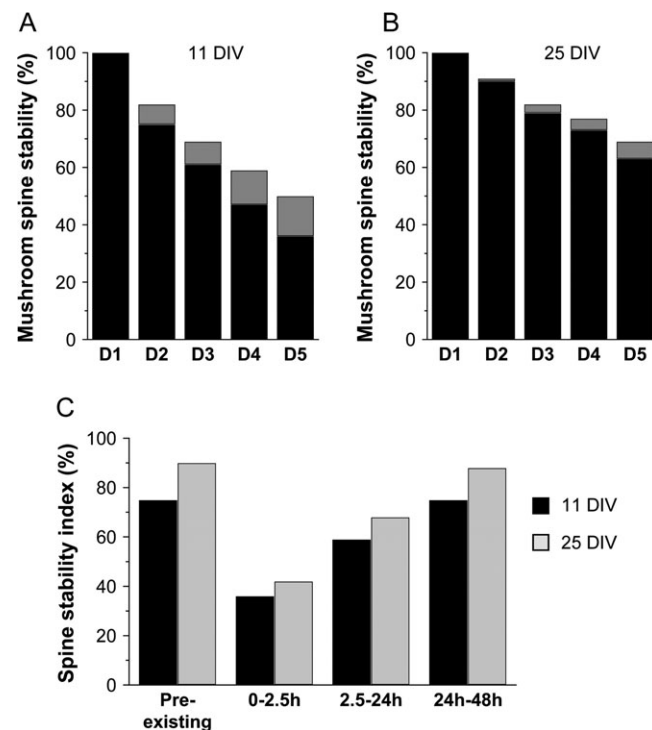


Figure 4. Changes in spine stability with development and spine age. (*A*) Fate of preexisting mushroom spines in 11 DIV slice cultures. Data are expressed as percent of initial mushroom spines still present as mushroom spines (black bars) or stubby spines (gray bars) on the next 4 consecutive days ($n = 10$ cells; 294 protrusions analyzed; calculated half-life: 4 days). (*B*) Same, but for mushroom spines in 25 DIV cultures ($n = 10$ cells; 239 protrusions analyzed; calculated half-life: 8 days). (*C*) Changes in stability of new spines analyzed as a function of spine age (0–2.5, 2.5–24, 24–48 h; $n = 14$ –408) in 11 DIV (black columns) and 25 DIV cultures (gray columns). Stability was defined as the proportion of new spines of the different ages that remained stable over the next 24 h. Note the marked increase in stability observed over the first 24 h.

repetitively imaged mushroom spines at time 0, 2.5, 24, 48, and 72 h on dendritic segments obtained from 3 to 7 different cells in 11 and 25 DIV cultures. We then calculated a stability index for newly formed spines of different ages (ages: 0–2.5, 2.5–24, 24–48 h) by measuring the proportion of them that were still present 24 h later. As shown in Figure 4C, in 11 DIV cultures, only 36% of the new mushroom spines formed during the 0- to 2.5-h interval ($n = 11$) remained after the first 24 h, indicating a very limited initial stability of very young, newly formed spines. We next considered the pool of new spines generated over the period 2.5–24 h ($n = 54$). From these, 59% were still present at 48 h, whereas for new spines, aged between 24 and 48 h, 75% of them persisted for the next 24 h (Fig. 4C), a value that is similar to that of preexisting spines. Thus, newly formed spines acquired the same stability as preexisting spines within a rather short period of time of about 24 h. Interestingly, very similar results were also obtained with 25 DIV slice cultures, despite the overall greater stability of preexisting spines at this developmental stage. Initially, newly formed spines aged 0–2.5 h showed a very high instability as only 42% of them persisted for

the next 24 h, but their stability then rapidly increased over the next 24 h to reach the overall stability of preexisting spines (Fig. 4C). This result therefore indicates the existence of a stabilization process that progressively increased the stability of newly formed spines over the first 24–48 h.

Spine Stability and Spine Head Size

We next investigated mechanisms that might contribute to this stabilization process by analyzing the possible role of the spine head size, which has previously been proposed to correlate with spine stability (Trachtenberg et al. 2002; Kasai et al. 2003; Holtmaat et al. 2006). In a first group of experiments, we simply measured the width of newly formed spines (age < 24 h) and compared it to the values obtained for 3 days stable spines. As shown in Figure 5B, newly formed mushroom spines were characterized by a significantly smaller spine head than stable ones (0.463 ± 0.011 vs. 0.539 ± 0.009 μm ; $n = 190$ – 379 ; $P < 0.001$), indicating that spine head enlargement is indeed part of a maturation process.

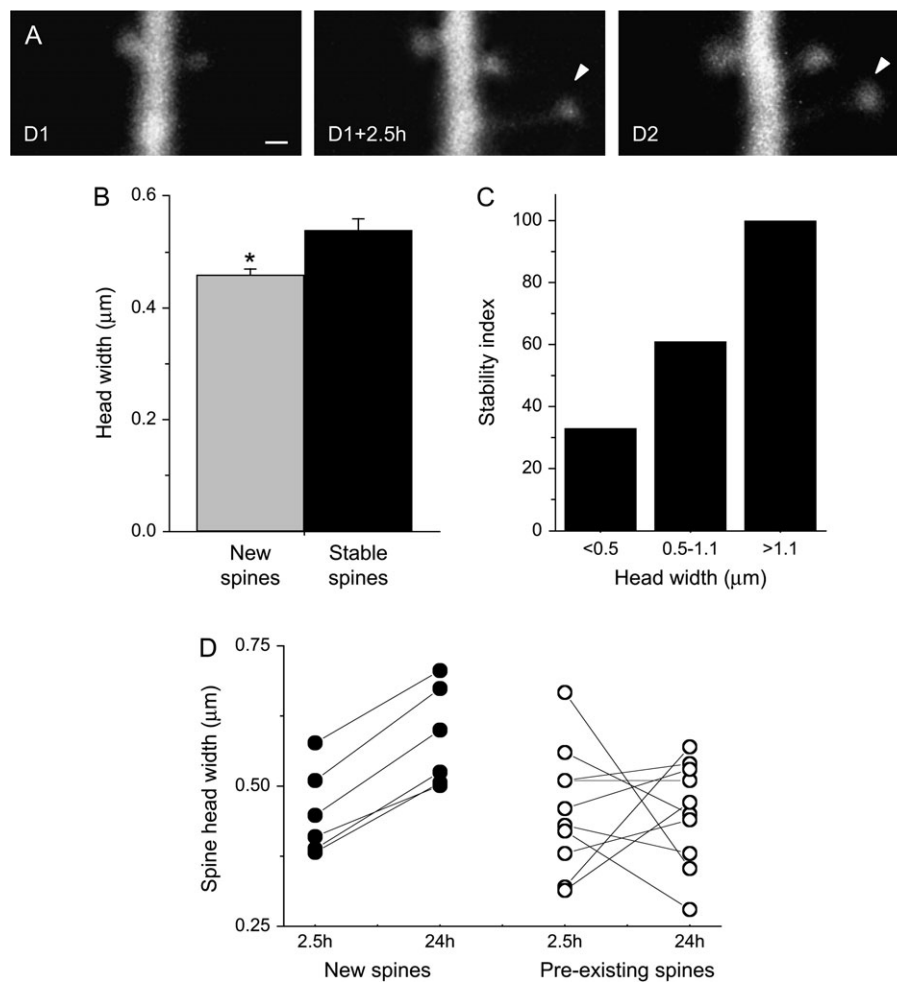


Figure 5. Stabilization of new spines is associated with an increase in spine head diameter. (A) Illustration of a newly formed mushroom spine showing an increase in head diameter over the first 24 h (scale bar: 0.5 μm). (B) Newly formed spines (age < 24 h) have smaller head width than stable spines (age > 72 h). Data are mean \pm standard error of the mean of measurements made on 190 new and 379 stable spines obtained from 16 cells ($P < 0.05$). (C) Stability of spines expressed as a function of the spine head diameter ($n = 6$ cells; 178 spines analyzed; $P < 0.001$). Stability was defined as the proportion of spines stable over 72 h. (D) Changes in spine head diameter measured over time in 6 newly formed mushrooms spines (filled circles) that showed a 24-h stability and 9 preexisting, stable spines located on the same dendritic segments (open circles).

Next we considered a pool of 178 mushroom spines obtained from 6 cells in 6 different slice cultures, imaged at 0 and 24 h and correlated their stability over 3 days with the size of their spine head. Three groups were generated that included small mushroom spines with a head width smaller than 0.5 μm ($n = 63$), medium size mushroom spines with head width between 0.5 and 1.1 μm ($n = 104$), and large mushroom spines with head widths larger than 1.1 μm ($n = 11$). These very large spines were rather infrequent and represented about 6% of all spines. As indicated in Figure 5C, there was a very clear correlation between spine head width and stability, with larger mushroom spines exhibiting a 100% probability of stability over 3 days. The size of the head is therefore a parameter that is directly correlated with and is even predictive of the stability of the spine.

To further assess how spine width changed over time in new mushroom spines, we carried out experiments in which we repeatedly assessed spine width of new mushroom spines over a period of 24 h. In 6 experiments, 30 new spines were detected within a 2.5-h period, and 6 out of those remained stable over the next 24 h. As illustrated in Figure 5A,D, all these new mushroom spines showed a marked increase in the size of their spine head over the next 24 h. In contrast, in a sample of 9 preexisting spines chosen in the vicinity of each of the new spines, the changes were variable with some spines enlarging and others becoming smaller but overall without global changes. In the 6 newly formed spines, the mean increase in spine head width averaged $29 \pm 2\%$, a result that contrasted with the mean value observed in preexisting spines ($0 \pm 13\%$; $n = 6-9$; $P < 0.05$). Furthermore, in a population of 190 preexisting mushroom spines taken from the same experiments, 43% showed an increase in the size of their head over 24 h. Considering a binomial law, the probability to observe 100% of enlarging spines out of 6 spines is less than 0.007. We thus conclude that the stabilization of a new spine is associated with an increase of its head width.

PSD Formation and Spine Stability

As the size of the spine head is known to correlate with the PSD size (Harris and Stevens 1989), we wondered whether the maturation period during which spine stability increased was related to the formation of the PSD. To address this question, we cotransfected CA1 pyramidal neurons with EGFP and PSD-95-DsRed2 in order to correlate spine formation and PSD formation in 11 DIV slice cultures. However, as PSD-95 transfection could have altered the basal properties of stability and turnover of spines (El-Husseini et al. 2000), we first measured spine turnover rate and 24 h stability of preexisting spines in EGFP, control, and PSD-95-transfected cells. For this, dendritic segments of 7 cotransfected neurons were imaged repetitively at times 0, 5, and 24 h and compared with control EGFP-transfected cells. These experiments showed no detectable difference for these values between cotransfected and control cells, indicating that PSD-95-DsRed2 transfection did not detectably affect the dynamics of spines and their stability within the time period analyzed. Then, we analyzed on 7 different cells the proportion of spines that exhibited or not a PSD staining. Figure 6A illustrates a dendritic segment from a cotransfected cell showing the presence of mushroom spines with and sometimes without a PSD staining. Overall, 88% of all mushroom spines analyzed ($n = 109$) exhibited a clear PSD

staining. In contrast, none of the 7 filopodia observed in these experiments showed PSD-95-DsRed2 labeling. These results suggested that a small fraction of spines were devoid of PSD. In order to verify this, we then also analyzed this possibility using electron microscopy, serial sections, and 3-dimensional reconstruction of spines. For this, 68 spines from 3 different cells were reconstructed from serial sections and, as illustrated in Figure 6B, 4 cases of spine-like protrusions without PSDs, could be observed, thereby confirming the existence in slice cultures of a small fraction of protrusions that are devoid of PSDs (see also Arellano et al. 2006). Note also that the spine illustrated in Figure 6B is also devoid of presynaptic partner.

Next we carried out experiments in which cotransfected cells were imaged repetitively at times 0, 5, 24 h, and the presence of a PSD in newly formed spines assessed. For this, cells were cotransfected with EGFP and PSD-95-DsRed2 and formation of new spines monitored by repetitive imaging. Then, at the end of the experiment, the presence of PSD-95-DsRed2 signal was assessed using a spinning-disk confocal system and a laser set at 568 nm. Figure 7 shows 2 examples of the results obtained in a pool of 10 similar experiments. In the first case, a new mushroom spine detected between 0 and 5 h (arrow-head) exhibited a clear PSD at 24 h (Fig. 7A), whereas in the second, a new spine aged 0-5 h (arrowhead) is devoid of PSD at 5 h (Fig. 7B). Quantitatively, the proportion of new mushroom spines (age < 24 h) that exhibited a PSD was significantly lower than that of preexisting ones ($60 \pm 10\%$, $n = 21$, vs. $90 \pm 3\%$, $n = 98$, $P < 0.05$; Fig. 7C), indicating that many new spines grew before expressing a PSD. In order to investigate more precisely the time course of PSD expression in newly formed spines, we then analyzed the proportion of spines with a PSD as a function of spine age. As illustrated in Figure 7D, no new mushroom spine aged less than 5 h exhibited a PSD ($n = 7$), although this proportion increased markedly for new mushrooms spines aged

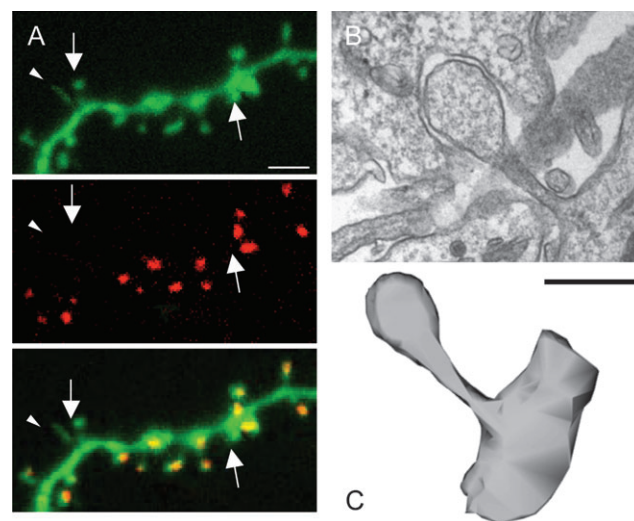


Figure 6. Absence of PSD-95-DsRed2 staining in a small fraction of dendritic spines. (A) Illustration of a dendritic segment of a CA1 pyramidal cell cotransfected with EGFP and PSD-95-DsRed2 showing that most dendritic spines visualized with EGFP (upper panel) exhibit a PSD-95-DsRed2 staining (middle panel) that colocalizes with the spine head (merged; lower panel). This contrasts with the absence of PSD-95-DsRed2 staining in 2 spines (arrows) and one filopodium (arrowhead). (B) Electron microscopic illustration of the existence of a spine-like protrusion that is devoid of PSD and even lacks a presynaptic partner. (C) Three-dimensional reconstruction of the spine illustrated in (B) (scale bar: A 2 μm ; B, C 0.5 μm).

5–19 h (69%, $n = 7$) and reached 86% for new spines aged 19–24 h ($n = 14$), a value close to that obtained for preexisting spines (aged > 24 h, $n = 98$). This result therefore indicated that newly formed spines did not initially express a PSD and that PSD expression occurred during the first 24 h after formation, a period which coincided in time with the stabilization of newly formed spines.

Role of Synaptic Activity in PSD Formation

We then wondered what was driving the formation of the PSD within the first 24 h and examined the possibility that synaptic activity could be involved. For this, we carried out a similar series of experiments as above in 11 DIV slice cultures, but in the presence of the glutamate receptor blockers D-AP5 and NBQX, applied in the culture medium throughout the exper-

iment (24 h). This treatment did not affect the stability of existing spines (proportion of stable spines: $78 \pm 4\%$ in control vs. $84 \pm 5\%$ in D-AP5/NBQX-treated cultures). However, as illustrated in Figure 8, blockade of synaptic activity markedly affected the expression of a PSD in newly formed spines. In the group of new spines aged 0–24 h, instead of finding $58 \pm 8\%$ of new spines expressing PSD-95–DsRed2 staining with synaptic activity blockade ($n = 19$ in control group and $n = 15$ in drugs treated group; $P < 0.001$). Furthermore, the capacity for stabilization of these new spines appeared to be reduced. In a set of 25 experiments, we measured the probability of stability over 24 h of new spines initially detected over a period of 5 h. Under control conditions, we found that $63 \pm 5\%$ of new spines (age < 5 h) were still present at 24 h, whereas this value decreased to $47 \pm 5\%$ following synaptic activity blockade

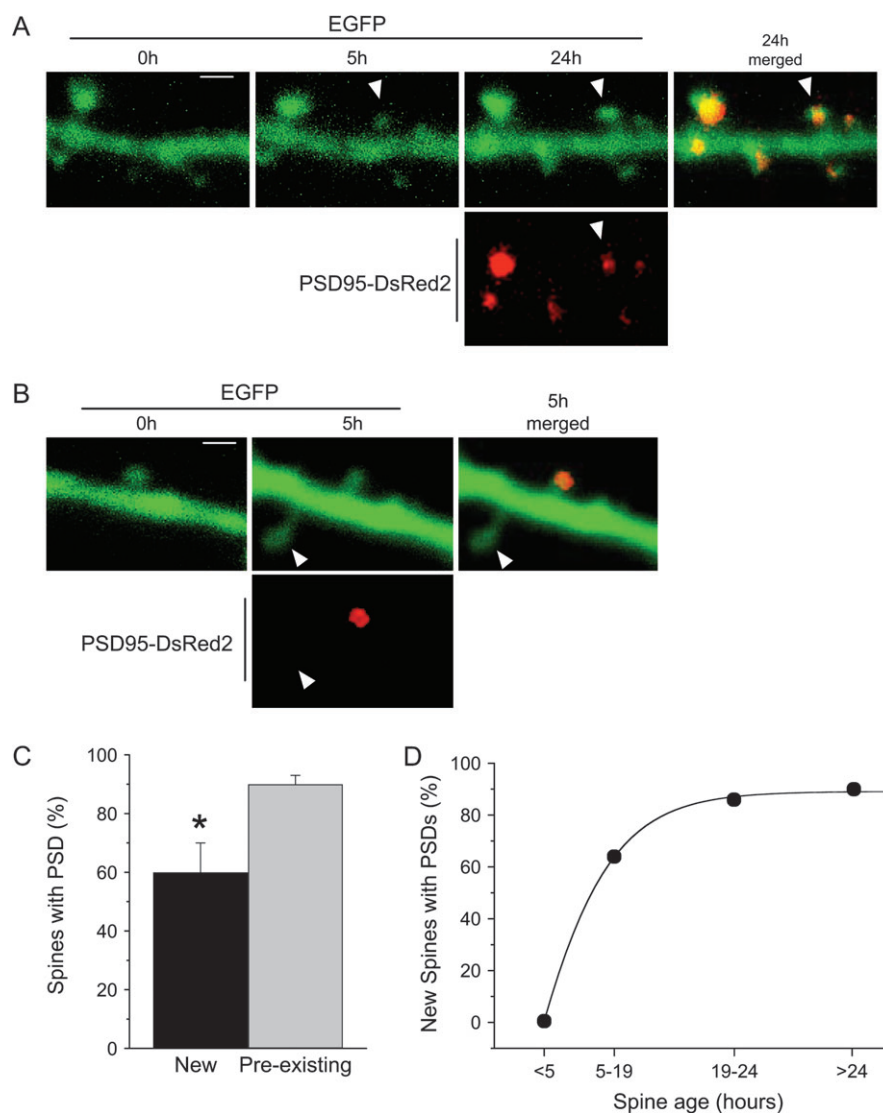


Figure 7. Time course of expression of PSD-95–DsRed2 in newly formed spines. (A) Illustration of a dendritic segment of a CA1 pyramidal cell cotransfected with EGFP and PSD-95–DsRed2 showing the formation of a new spine observed at time 5 h (arrowhead) that expressed PSD-95–DsRed2 staining at 24 h (lower panel), colocalizing with the spine head. (B) Illustration of a dendritic segment of a CA1 pyramidal cell cotransfected with EGFP and DsRed–PSD-95 showing the formation of a new spine observed at time 5 h (arrowhead), which does not present PSD-95–DsRed2 staining at this time point (lower panel). (C) Quantitative analysis of the proportion of newly formed spines aged less than 24 h and preexisting spines aged more than 24 h that expressed PSD-95–DsRed2. Data are mean \pm standard error of the mean ($n = 21$ and $n = 98$ for new and preexisting spines, respectively; $P < 0.05$). (D) Time course of PSD-95–DsRed2 expression in newly formed spines as a function of the age of the spines (0–5 h: $n = 7$; 5–19 h: $n = 14$; 19–24 h: $n = 7$; >24 h: $n = 98$; scale bar: A and B 0.5 μ m).

($n = 23\text{--}32$; $P < 0.05$). These results therefore suggest a role for synaptic activity in regulating expression of the PSD and stabilization of newly formed spines.

Discussion

By analyzing dendritic spine turnover in developing hippocampal organotypic slice cultures, we provide here important new insights into the mechanisms that contribute to the formation and stabilization of new protrusions. First, we find that the high rate of protrusion formation that underlies synaptic development in slice cultures is surprisingly poorly effective, as most new protrusions (86–90%) are only transient and do not result in the formation of stable dendritic spines. This is particularly the case for filopodia, which, although numerous, almost always failed to generate stable mushroom spines. Second, this early elimination of newly formed spines appears to be characteristic of the spine formation process, independently of the developmental stage of the tissue, and to occur during a critical stabilization period of about 24 h. Interestingly, we find that this stabilization is directly correlated in its time course with an enlargement of the spine head and the expression of a PSD, and furthermore that expression of the PSD is regulated by synaptic activity. All together these results are consistent with a model where synaptic development proceeds through a marked, non-specific growth of dendritic protrusions, followed by the stabilization of a small number of spines through a process

that involves the expression of a PSD through a mechanism that is driven by synaptic activity (Fig. 9).

This interpretation is in agreement with a number of existing observations regarding spine properties or plasticity. First, the turnover rate observed here in hippocampal slice cultures not only compares well with the results reported in previous, shorter time-lapse experiments (Engert and Bonhoeffer 1999; Jourdain et al. 2003; Nagerl et al. 2004) but also falls within the variability reported in the cortex of living mice (Grutzendler et al. 2002; Trachtenberg et al. 2002; Holtmaat et al. 2005; Majewska et al. 2006). In particular, the values obtained in 3- to 4-week-old cultures appear to be in range quite similar to that reported in the barrel cortex of young, adult mice (Grutzendler et al. 2002). Furthermore, the stability of spines in 25 DIV slice cultures is consistent with what is reported in young mice (Grutzendler et al. 2002; Trachtenberg et al. 2002). Also, we find that this turnover rate is strongly developmentally regulated in slice cultures as was reported *in vivo* (Holtmaat et al. 2005; Zuo, Lin, et al. 2005). These similarities add therefore to the important correlations found otherwise in terms of spine density, spine type distribution, spine shape, or synaptic properties and function reported between hippocampal organotypic slice cultures and hippocampal or cortical tissue (Gahwiler et al. 1997; Toni et al. 2001), strengthening the likely physiological relevance of the present results.

Although the existence of a developmental regulation of protrusion formation in slice cultures is of interest, a probably more surprising result is the high proportion of these new protrusions that are rapidly eliminated. This process seems to differ from the usual concept of pruning that involves a competitive elimination of existing spines or synapses (Segal 2005; Zuo, Lin, et al. 2005). Here protrusions appear to be eliminated even before they have a chance to form a synaptic contact and express PSD-95. This conclusion is based both on confocal analyses of the lack of expression of tagged PSD-95 in very young, newly formed spines and electron microscopic observations of spine-like protrusions that are devoid of PSDs or sometimes even lack a presynaptic partner in their immediate vicinity (Fig. 6; Arellano et al. 2006). It would seem therefore that the growth of these new protrusions initially occurs without presynaptic involvement, a conclusion that is in agreement with and extend previous and recent *in vivo* observations (Marrs et al. 2001; Knott et al. 2006). As shown in this study, the decision between elimination and stabilization appears to be made within the first 24 h, a time course that does not seem to vary with development, because the phenomenon was similar in 11 and 25 DIV cultures, despite important

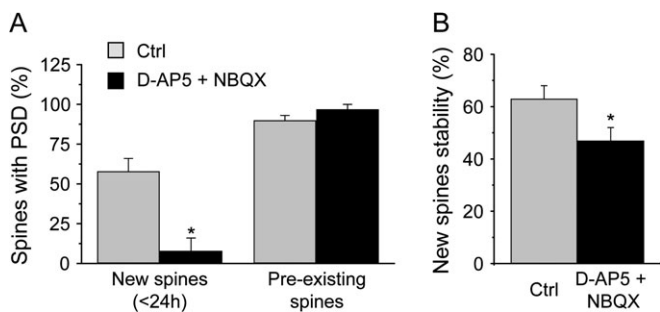


Figure 8. Synaptic activity regulates the expression of PSD-95-DsRed2 staining and the stabilization of a new spine. (A) Proportion of newly formed spines, aged less than 24 h, and stable, preexisting spines that expressed PSD-95-DsRed2 under control conditions (gray columns) and in the presence of D-AP5 (50 μ M) and NBQX (10 μ M; black columns; $*P < 0.001$). (B) Stability of newly formed spines, measured as the probability of new spines aged 0–5 h to be still present 24 h later under control conditions (gray column) and in the presence of D-AP5 and NBQX ($n = 23\text{--}32$; $*P < 0.05$).

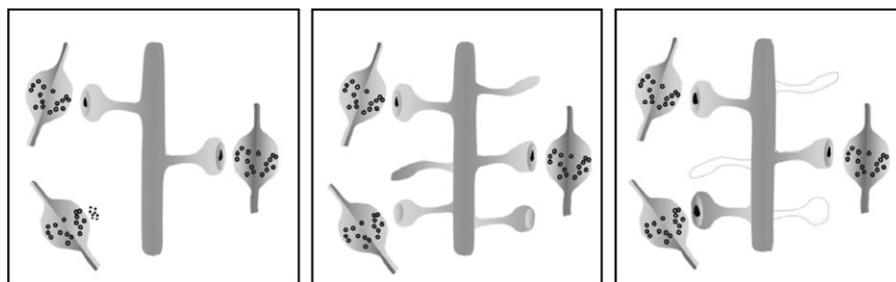


Figure 9. Schematic diagram summarizing the mechanisms of synaptic development likely to occur in hippocampal slice cultures. Spine turnover involves the growth of many new protrusions extending in a nonspecific manner (middle panel). Among these, most of them will be rapidly eliminated within 24 h, whereas the few of them that will experience synaptic activity will be stabilized through expression of a PSD (right panel).

differences in the overall stability of dendritic spines. This timing therefore strongly suggests a general mechanism that regulates the stabilization of new protrusions over the first 24 h. Curiously, this stabilization was found to be much more likely for new protrusions appearing as mushroom spines than for filopodia. Filopodia have been proposed in many previous studies to represent precursors of mushroom spines and there is indeed evidence, also observed here, that filopodia can express PSDs and transform into mushroom spines (Ziv and Smith 1996; Fiala et al. 1998; Marrs et al. 2001). However, although we cannot rule out a role of filopodia during the very first stages of neuron development, the current results indicate that their contribution to the remodeling of synaptic networks remains marginal, a phenomenon also pointed out in *in vivo* experiments (Zuo, Lin, et al. 2005). All together therefore, one can conclude that the role of filopodia is probably extremely limited in promoting the development of stable synaptic contacts.

What then accounts for the stabilization of new protrusions? Our present data indicate that both an enlargement of the spine head and the acquisition of a PSD coincide in time with an increased stability. Considering that in spines, the size of the head, the size of the PSD, the expression of receptors, and even the level of presynaptic activity show strong correlations (Harris and Stevens 1989; Nusser et al. 1998; Takumi et al. 1999; El-Husseini et al. 2000; Knott et al. 2006), it could very well be that stability is acquired through functionality. A key observation made here supporting this interpretation is that PSD expression in newly formed spines was markedly reduced upon blockade of postsynaptic glutamate receptors. Although it remains unclear in these experiments whether it is a decrease in release or the direct blockade of receptors that prevents expression of a PSD, these results still clearly indicate that synaptic activity might precede formation of a detectable PSD and even be required for its formation. Activity, by promoting the expression of a PSD, might thus exert a stabilizing influence on the new spine. Indeed, most stable spines expressed a PSD, and spines with a PSD have never been reported to lack a presynaptic partner on electron microscopic analyses. Also this interpretation is in line with recent observations made on filopodia indicating the existence of calcium transient on these protrusions even in the absence of PSDs in most of them (Lohmann et al. 2005). This is also consistent with the recent evidence indicating that spine dynamics and turnover have their functional correlate in cortical slices through the rapid appearance and disappearance of synaptic signals, indicating that functionality of new spines may be acquired quite quickly (Le Be and Markram 2006). New protrusions might therefore sense transmitter release before expressing a clear PSD and a main function of the PSD might then be stabilization of the protrusion. PSDs are indeed highly dynamic structures that can be quickly inserted in membranes and promote in this way protrusion stability (Bresler et al. 2001; Marrs et al. 2001; Prange and Murphy 2001) through interactions via a variety of molecular constituents (Ethell and Pasquale 2005; Garner et al. 2006). Very recent work by Gray et al. (2006) does indeed show a high level of trafficking of PSD-95 in and out of PSDs. Finally, it is interesting that the mechanisms described here to result in stabilization of newly formed spines appear to have strong similarities with the changes that have been associated with the increase in synaptic strength that characterizes LTP. An enlargement of the spine head with increased expression of AMPA receptors and modifications of PSDs have also been proposed to result in a better stability of activated

spines (Matsuzaki et al. 2001, 2004). Also synaptic activity through the continuous, spontaneous release of transmitter was shown to be critical for the maintenance of dendritic spines (McKinney et al. 1999). All together, these results point to the importance of transmitter release and synaptic activity as probable key regulators of the stabilization of newly formed protrusions through incorporation, enlargement, or maintenance of PSDs.

It remains however unclear what are the molecular signals that effectively drive the incorporation of the PSD in new spines. They probably include signaling cascades mediated by postsynaptic receptor activation, but there might be also a role for growth factors or even cell-cell signaling molecules (Ethell and Pasquale 2005). In this regard, the approach used here clearly opens new possibilities to directly address some of these critical questions. It is likely indeed that the mechanisms regulating this early step of stabilization of newly formed protrusions will have a profound impact on the development of a functional synaptic network and possibly be involved in some of the defects in spine morphogenesis that have recently been associated with different forms of mental diseases (Halpain et al. 2005; Grossman et al. 2006).

Notes

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References

- Arellano JI, Espinosa A, Fairen A, Yuste R, Defelipe J. 2006. Non-synaptic dendritic spines in neocortex. *Neuroscience*. 145:464–469.
- Bresler T, Ramati Y, Zamorano PL, Zhai R, Garner CC, Ziv NE. 2001. The dynamics of SAP90/PSD-95 recruitment to new synaptic junctions. *Mol Cell Neurosci*. 18:149–167.
- Buchs PA, Stoppini L, Muller D. 1993. Structural modifications associated with synaptic development in area CA1 of rat hippocampal organotypic cultures. *Brain Res Dev Brain Res*. 71:81–91.
- Collin C, Miyaguchi K, Segal M. 1997. Dendritic spine density and LTP induction in cultured hippocampal slices. *J Neurophysiol*. 77:1614–1623.
- El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS. 2000. PSD-95 involvement in maturation of excitatory synapses. *Science*. 290:1364–1368.
- Engert F, Bonhoeffer T. 1999. Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature*. 399:66–70.
- Ethell IM, Pasquale EB. 2005. Molecular mechanisms of dendritic spine development and remodeling. *Prog Neurobiol*. 75:161–205.
- Fiala JC, Feinberg M, Popov V, Harris KM. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J Neurosci*. 18:8900–8911.
- Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. 1997. Organotypic slice cultures: a technique has come of age. *Trends Neurosci*. 20:471–477.
- Garner CC, Waites CL, Ziv NE. 2006. Synapse development: still looking for the forest, still lost in the trees. *Cell Tissue Res*. 326:249–262.
- Gray NW, Weimer RM, Bureau I, Svoboda K. 2006. Rapid redistribution of synaptic PSD-95 in the neocortex *in vivo*. *PLoS Biol*. 4:e370.
- Grossman AW, Elisescu NM, McKinney BC, Greenough WT. 2006. Hippocampal pyramidal cells in adult Fmr1 knockout mice exhibit an immature-appearing profile of dendritic spines. *Brain Res*. 1084:158–164.

- Grutzendler J, Kasthuri N, Gan WB. 2002. Long-term dendritic spine stability in the adult cortex. *Nature*. 420:812-816.
- Halpain S, Spencer K, Graber S. 2005. Dynamics and pathology of dendritic spines. *Prog Brain Res*. 147:29-37.
- Harris KM, Stevens JK. 1989. Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. *J Neurosci*. 9:2982-2997.
- Holtmaat A, Wilbrecht L, Knott GW, Welker E, Svoboda K. 2006. Experience-dependent and cell-type-specific spine growth in the neocortex. *Nature*. 441:979-983.
- Holtmaat AJ, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, Svoboda K. 2005. Transient and persistent dendritic spines in the neocortex in vivo. *Neuron*. 45:279-291.
- Jourdain P, Fukunaga K, Muller D. 2003. Calcium/calmodulin-dependent protein kinase II contributes to activity-dependent filopodia growth and spine formation. *J Neurosci*. 23:10645-10649.
- Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H. 2003. Structure-stability-function relationships of dendritic spines. *Trends Neurosci*. 26:360-368.
- Kennedy MJ, Ehlers MD. 2006. Organelles and trafficking machinery for postsynaptic plasticity. *Annu Rev Neurosci*. 29:325-362.
- Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K. 2006. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat Neurosci*. 9:1117-1124.
- Le Be JV, Markram H. 2006. Spontaneous and evoked synaptic rewiring in the neonatal neocortex. *Proc Natl Acad Sci USA*. 103:13214-13219.
- Lohmann C, Finski A, Bonhoeffer T. 2005. Local calcium transients regulate the spontaneous motility of dendritic filopodia. *Nat Neurosci*. 8:305-312.
- Majewska AK, Newton JR, Sur M. 2006. Remodeling of synaptic structure in sensory cortical areas in vivo. *J Neurosci*. 26:3021-3029.
- Maletic-Savatic M, Malinow R, Svoboda K. 1999. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science*. 283:1923-1927.
- Marrs GS, Green SH, Dailey ME. 2001. Rapid formation and remodeling of postsynaptic densities in developing dendrites. *Nat Neurosci*. 4:1006-1013.
- Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H. 2001. Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nat Neurosci*. 4:1086-1092.
- Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H. 2004. Structural basis of long-term potentiation in single dendritic spines. *Nature*. 429:761-766.
- McKinney RA, Capogna M, Durr R, Gahwiler BH, Thompson SM. 1999. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci*. 2:44-49.
- Nagerl UV, Eberhorn N, Cambridge SB, Bonhoeffer T. 2004. Bidirectional activity-dependent morphological plasticity in hippocampal neurons. *Neuron*. 44:759-767.
- Nicoll RA. 2003. Expression mechanisms underlying long-term potentiation: a postsynaptic view. *Philos Trans R Soc Lond B Biol Sci*. 358:721-726.
- Nicoll RA, Tomita S, Bredt DS. 2006. Auxiliary subunits assist AMPA-type glutamate receptors. *Science*. 311:1253-1256.
- Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P. 1998. Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron*. 21:545-559.
- Prange O, Murphy TH. 2001. Modular transport of postsynaptic density-95 clusters and association with stable spine precursors during early development of cortical neurons. *J Neurosci*. 21:9325-9333.
- Segal M. 2005. Dendritic spines and long-term plasticity. *Nat Rev Neurosci*. 6:277-284.
- Stepanyants A, Hof PR, Chklovskii DB. 2002. Geometry and structural plasticity of synaptic connectivity. *Neuron*. 34:275-288.
- Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods*. 37:173-182.
- Takumi Y, Ramirez-Leon V, Laake P, Rinvik E, Ottersen OP. 1999. Different modes of expression of AMPA and NMDA receptors in hippocampal synapses. *Nat Neurosci*. 2:618-624.
- Toni N, Buchs PA, Nikonenko I, Bron CR, Muller D. 1999. LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature*. 402:421-425.
- Toni N, Buchs PA, Nikonenko I, Povelaitite P, Parisi L, Muller D. 2001. Remodeling of synaptic membranes after induction of long-term potentiation. *J Neurosci*. 21:6245-6251.
- Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K. 2002. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature*. 420:788-794.
- Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB. 1998. Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature*. 391:892-896.
- Turrigiano GG, Nelson SB. 2004. Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci*. 5:97-107.
- Yuste R, Bonhoeffer T. 2004. Genesis of dendritic spines: insights from ultrastructural and imaging studies. *Nat Rev Neurosci*. 5:24-34.
- Ziv NE, Smith SJ. 1996. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron*. 17:91-102.
- Zuo Y, Lin A, Chang P, Gan WB. 2005. Development of long-term dendritic spine stability in diverse regions of cerebral cortex. *Neuron*. 46:181-189.
- Zuo Y, Yang G, Kwon E, Gan WB. 2005. Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. *Nature*. 436:261-265.