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Development of Long-Term Dendritic Spine Stability in Diverse Regions of Cerebral Cortex

Report

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Summary

Synapse formation and elimination occur throughout life, but the magnitude of such changes at distinct developmental stages remains unclear. Using transgenic mice overexpressing yellow fluorescent protein and transcranial two-photon microscopy, we repeatedly imaged dendritic spines on the apical dendrites of layer 5 pyramidal neurons. In young adolescent mice (1-month-old), 13%–20% of spines were eliminated and 5%–8% formed over 2 weeks in barrel, motor, and frontal cortices, indicating a cortical-wide spine loss during this developmental period. As animals mature, there is also a substantial loss of dendritic filopodia involved in spinogenesis. In adult mice (4–6 months old), 3%–5% of spines were eliminated and formed over 2 weeks in various cortical regions. Over 18 months, only 26% of spines were eliminated and 19% formed in adult barrel cortex. Thus, after a concurrent loss of spines and spine precursors in diverse regions of young adolescent cortex, spines become stable and a majority of them can last throughout life.

Introduction

Changes in synaptic connections are essential for the development and function of the nervous system. In the developing mammalian cerebral cortex, synaptic density increases rapidly before birth but decreases substantially from late postnatal development until puberty (e.g., Huttenlocher, 1990; Markus and Petit, 1987; Rakic et al., 1986). In adulthood, the number of synapses appears relatively constant until the late stages of life (Masliah et al., 1993; Peters et al., 1998; Rakic et al., 1986). Although synaptic density becomes stabilized as animals mature, many lines of evidence indicate that synaptic connectivity in adulthood can be continuously modified by learning-related tasks and environmental factors (e.g., Buonomano and Merzenich, 1998; Darian-Smith and Gilbert, 1994; Fox, 2002; Grossman et al., 2002; Jones et al., 1997; Knott et al., 2002). However, it remains largely unknown to what degree changes in synaptic connectivity in vivo represent the formation and elimination of synapses or the modification of the strength of existing synapses.

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Given the complexity and variability of synaptic connections in the nervous system, it is crucial to determine the nature and degree of synaptic dynamics by imaging individual synapses repeatedly in the same animal (Lichtman et al., 1987; Purves et al., 1987). Recent technological advances, including two-photon microscopy and the generation of transgenic mice overexpressing green fluorescent protein (GFP) and its spectral variants (e.g., CFP, YFP), have made it possible to image individual dendritic arbors and spines over long periods of time in living animals (Denk et al., 1990; Feng et al., 2000; Grutzendler et al., 2002; Mizrahi and Katz, 2003; Niell et al., 2004; Trachtenberg et al., 2002). Because dendritic spines are the postsynaptic sites of the vast majority of excitatory synapses in the cortex, their changes serve as a good indicator of synaptic change. Recent studies have shown that spines in the mouse primary visual cortex are plastic during young adolescence and become remarkably stable in adulthood, with ~4% turnover per month (Grutzendler et al., 2002). However, a different study in the mouse barrel cortex found that adult spines are highly dynamic, with ~20% turnover per day (Trachtenberg et al., 2002). Although transgenic lines, animal ages, and methodologies differ in the two studies, these apparently contradictory results raise the possibility that various cortical regions may demonstrate different spine dynamics.

In this work, we used a transcranial two-photon imaging technique to follow individual spines of layer 5 pyramidal neurons over extended periods of time in different cortical regions (barrel, primary motor, and frontal cortex) at distinct developmental stages. We found that in adulthood (>4 months of age), 3%–5% of spines turn over over 2 weeks in all the cortical regions examined. Remarkably, a majority (>70%) of spines can last over 18 months in the barrel cortex. This lifelong adult spine stability is achieved by eliminating spines and the precursors of spines in the young adolescent cortex.

Results

YFP Expression in Different Cortical Regions of H-Line Transgenic Mice

To determine spine dynamics in various cortical regions, we used the YFP-H-line transgenic mice in which YFP is expressed in a small subset of cortical neurons (Feng et al., 2000). We found that at 1 month of age, YFP expression was mainly restricted to layer 5 and 6 pyramidal neurons and the ratio of YFP-expressing cells within these two layers varied among cortical regions (Figure 1A and Figure S1 in the Supplemental Data available with this article online). As the animal ages, the number of YFP-expressing cells in these cortical areas increases gradually. While only few cells express YFP in the first postnatal week, the densities of YFP-positive cells in layer 5 and 6 increase to 3–10 cells/200 μm^3 at 1 month of age and ~20 cells/200 μm^3 at 4–6 months of age. YFP expression can also be

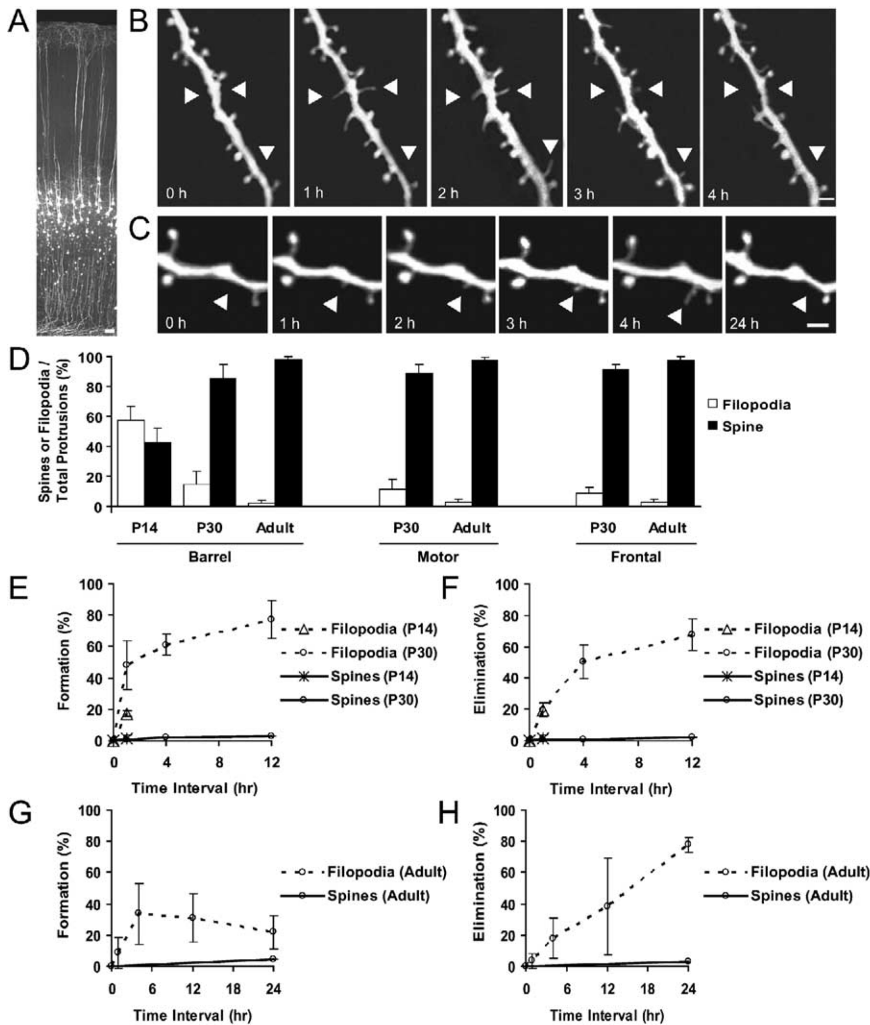

Figure 1. Transcranial Two-Photon Imaging Shows Dynamic Dendritic Filopodia in Young and Adult Mice

(A) A subset of layer 5 pyramidal cells express YFP and extend their apical dendrites to layer 1 in barrel cortex. Some layer 6 cells also express YFP but do not project to layer 1. Scale bar, 50 μm.

(B and C) Time-lapse imaging (1 hr intervals) in 1-month-old (B) and adult (C) mice reveals that filopodia (e.g., arrowheads) undergo rapid extension and retraction, whereas spines on the same dendritic branches remain stable. Scale bars, 2 μm.

(D) Percentage of spines and filopodia (number of spines or filopodia/total protrusions) in different cortical regions in young and adult animals. Much fewer filopodia exist in adult than in young animals.

(E–H) Percentage of filopodia and spines formed (e.g., number of spines formed/preexisting number of spines) and eliminated over a period of 12–24 hr in young (E and F) and adult (G and H) animals. Data are presented as mean ± SD.

found in a small percentage (<8%) of layer 2/3 neurons in the adult barrel cortex. Although there are more YFP-positive cells in layer 6 than in layer 5 of barrel, frontal, and visual cortices, only layer 5 neurons project to superficial layer 1 (Figure 1A and Figure S1; Kasper et al., 1994; Lubke and Albus, 1989). In the following study, we only imaged and analyzed dendrites located within the first 100 μm of the cortical surface. Therefore, spine dynamics in this study are mainly related to the apical dendrites of layer 5 pyramidal cells.

Dendritic Filopodia and Spines Have Very Different Short-Term Dynamics

Many lines of evidence suggest that dendrites contain two kinds of protrusions: filopodia and spines. Unlike spines, filopodia are long and thin protrusions without a bulbous head, many of which do not form synaptic contact with presynaptic axons (Dailey and Smith, 1996; Fiala et al., 1998; Yuste and Bonhoeffer, 2004; Ziv and Smith, 1996). Consistent with their role in synaptogenesis (Dailey and Smith, 1996; Fiala et al., 1998; Ziv and Smith, 1996), filopodia are highly abundant during early postnatal development when extensive synapse formation occurs. In barrel cortex, for example, more than half of dendritic protrusions were filopodia at 2 weeks of age (Figure 1D; 54.2% ± 7.2%, 569 protrusions, 5 mice; see Grutzendler et al., 2002 and also the Supplemental Data for classification of filopodia and spines). As animals mature and the peak of synaptogenesis is over, the number of filopodia decreases dra-

matically. In young adolescent mice at 1 month of age, ~10% of dendritic protrusions in different cortical regions are filopodia, while the rest are spines (Figures 1B and 1D). In adult mice, the percentage of filopodia further reduces to only 2%–3% across all the cortical regions studied, significantly lower than that observed in young mice (Figures 1C and 1D).

Consistent with previous studies in young adolescent visual cortex (Grutzendler et al., 2002), we found that in barrel cortex at postnatal day 14, filopodia were highly dynamic, with 17.4% ± 1.9% formed and 19.8% ± 4.4% eliminated over 1 hr (Figures 1E and 1F; 160 filopodia, 3 mice). Similarly, at 1 month of age, 48.3% ± 15.8% of filopodia were formed and 19.4% ± 4.9% were eliminated within 1 hr (Figures 1B, 1E, and 1F; 247 filopodia, 6 mice). Within a 4 hr interval, 61.2% ± 6.6% of filopodia were formed and 50.4% ± 10.6% were eliminated (Figures 1B, 1E, and 1F; 119 filopodia, 4 mice at 1 month of age). Time-lapse imaging every hour over a total of 4 hr showed that 9.9% ± 3.7% of filopodia disappeared and reappeared at the same location, further indicating the dynamic nature of these protrusions. In contrast to filopodia, 1.8% ± 0.6% of spines were formed and 0.8% ± 0.9% were eliminated within 4 hr (859 spines, 6 mice at 1 month of age). Thus, unlike the vast majority of spines, filopodia in the young cortex are highly dynamic structures with a lifetime on the order of several hours.

Although filopodia are scarce in adulthood, they also underwent rapid changes over hours. In barrel cortex, for example, 8.8% ± 10.2% of them were formed and 3.5% ± 4.8% were eliminated within a 1 hr interval. Over 4 hr, 33.3% ± 19.4% of filopodia were formed and 17.5% ± 12.9% were eliminated (Figures 1C, 1G, and 1H; 57 filopodia, 5 mice). In contrast, nearly all spines in the same area were stable over hours to days (Figures 1C, 1G, 1H, and 3O). Thus, similar to observations in young cortex, adult filopodia and spines also have very different dynamics.

Dendritic Filopodia Are Intermediaries in Spine Formation and Elimination

While the majority of filopodia underwent rapid turnover in young adolescent barrel cortex (1 month of age), a small percentage (14.8% ± 2.8%) formed a bulbous head and persisted over 4 hr (Figures 2A and 2B; 119 filopodia, 4 mice). In addition, ~40% of these newly formed spine-like protrusions persisted over 24 hr, and ~20% lasted over 48 hr (Figure 2D). These protrusions are morphologically indistinguishable from preexisting spines. Similarly, in adulthood, we found that 4 out of 57 filopodia formed a bulbous head within 4 hr (Figure 2C, 5 mice) and 1 of them persisted after 24 hr. These observations suggest that filopodia serve as precursors of spines that can last over days in both youth and adulthood.

It is important to note that although filopodia serve as spine precursors, spine formation appears to be a highly selective process. In young adolescent mice at 1 month of age, the total number of filopodia generated over 1 day was at least one-third of the total number of preexisting spines (based on the observation that

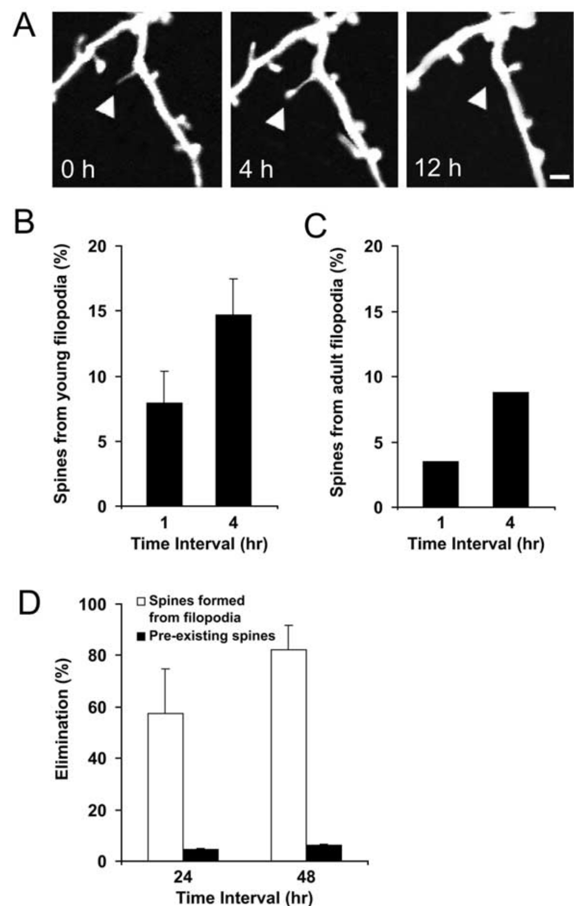

Figure 2. A Small Percentage of Filopodia Is Converted into Long-Lasting Spines in Barrel Cortex

(A) Time-lapse imaging over 12 hr shows that one filopodium is first converted to a spine-like protrusion within 4 hr and disappears by 12 hr. (B and C) Percentage of filopodia that become spines over 1 and 4 hr in both young (B) and adult mice (C). (D) Percentage of eliminated spines that are newly formed from filopodia is significantly higher than that of preexisting spines over 1–2 days. Data are presented as mean ± SD. Scale bars, 2 μm.

>50% filopodia turnover over 4 hr and the ratio of filopodia to spine is ~1:9). Even though a small percentage of these filopodia were converted into spine-like protrusions, these newly formed spines were largely eliminated over 1–2 days, while the vast majority of preexisting spines were stable (Figures 2B and 2D). Overall, only ~6% (14.8% × 40%) of filopodia eventually formed a spine lasting 1 day, and <3% (14.8% × 20%) formed a spine lasting 2 days.

In addition to mediating spine formation, filopodia may also be involved in the process of spine elimination. When imaged multiple times at 0, 4, and 12 hr, 15 out of 745 spines in young animals (n = 6) were eliminated by the end of the observation period. Seven of these 15 eliminated spines assumed a filopodia-like structure by the 4 hr time point before they disappeared within the next 8 hr. The other eight spines were present at 4 hr, but disappeared between 4 and 12 hr. Thus, at least some, if not all, eliminated spines are transiently converted to filopodia before elimination.

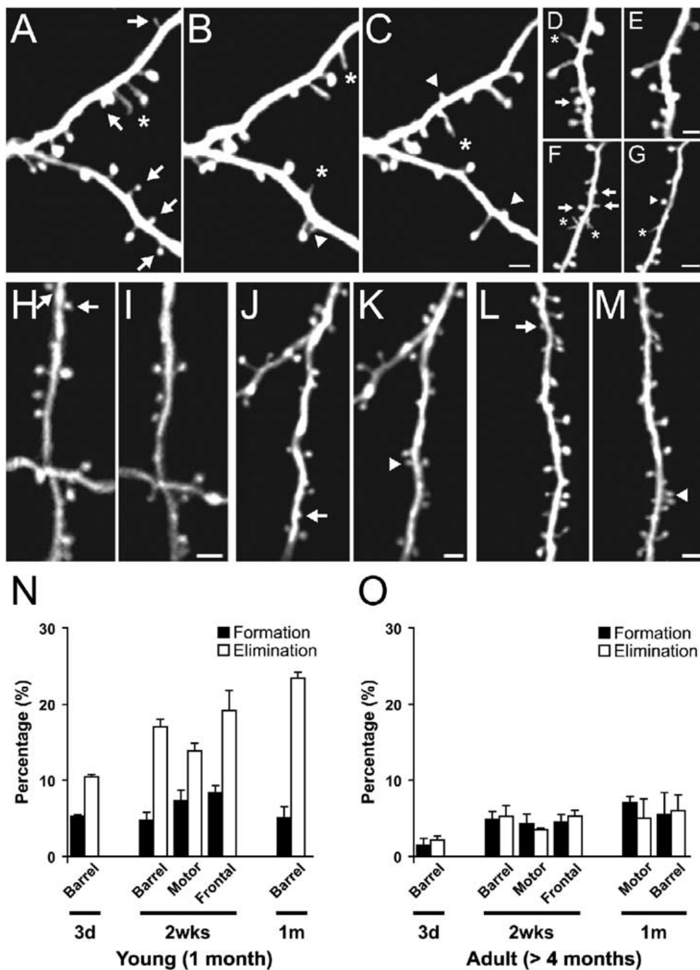

Figure 3. Dendritic Spines Become Stable in Adulthood after Substantial Spine Elimination during Young Adolescence

(A–G) In young adolescent mice (1 month of age), repeated imaging of dendritic branches over a 2 week interval reveals a predominant elimination of spines and filopodia in different cortical regions: barrel (A–C), motor (D and E), and frontal (F and G). (H–M) In adult mice (>4 months), dendritic branches imaged 2–4 weeks apart show that the vast majority of spines are at the same locations in different regions of adult cortex: frontal ([H and I], 2 weeks), motor ([J and K], 2 weeks), barrel ([L and M], 4 weeks). (N and O) Percentage of spines formed and eliminated over various time intervals in different cortical regions in young mice at 1 month of age (N) and adults (O). Arrows indicate spines that are eliminated in the next view, while arrowheads indicate spines that are formed since the previous view. Asterisks indicate filopodia. Data are presented as mean ± SD. Scale bars, 2 μm.

Consistent with the role of filopodia in spine formation and elimination, we found that the rate of spine turnover correlates with the abundance of filopodia. In young adolescent barrel cortex in which ~10% of dendritic protrusions are filopodia, 5.3% ± 0.2% of spines were formed and 10.5% ± 0.3% were eliminated over 3 days (Figure 3N; 748 spines, 3 mice). In adult barrel cortex in which 2%–3% of protrusions are filopodia, 1.5% ± 0.9% spines were formed and 2.1% ± 0.5% spines were eliminated over 3 days (Figure 3O; 454 spines, 3 mice). Together, these observations suggest that filopodia are actively involved in spinogenesis and that their abundance contributes to heightened spine turnover rates in young adolescent cortex.

More Spines and Filopodia Are Eliminated than Formed in Young Adolescent Cortex

To examine long-term changes of spines, we repeatedly imaged identified dendritic segments over a 2 week interval in layer 1 of barrel, motor, and frontal cortices in young adolescent mice (1 month of age). While the vast majority of filopodia disappeared from their original location over 2 weeks, more than 80% of spines persist in various cortical regions (Figures 3A–3G and 3N; barrel: 856 spines; motor: 410 spines; frontal: 392 spines; 3 mice for each cortical region). In addition, the rate of spine formation was significantly lower than that of spine elimination (Figure 3N; $p < 0.01$ in all the regions, paired t test), demonstrating that spine number decreases during this period of development. Furthermore, there is a significant reduction in the number of filopodia in different cortical regions from 1 to 1.5 months of age. Over this 2 week interval in barrel cortex, for example, the percentage of filopodia is reduced from 14.4% ± 9.2% to 5.9% ± 2.1%, which is close to that in adulthood (2.1% ± 2.0%). These results were comparable to those shown previously in the primary visual cortex over the same time interval (Grutzendler et al., 2002). Therefore, as animals mature, there is a concurrent loss of dendritic spines and filopodia in diverse regions of young adolescent cerebral cortex.

Dendritic Spines Become Progressively Stabilized from Young Adolescence to Adulthood

To determine the degree of spine stability in adult cortex (4–6 months of age), we first compared spine turnover rates in adult primary motor, frontal, and barrel cortices over a 2 week interval. We found that 3.5% ±

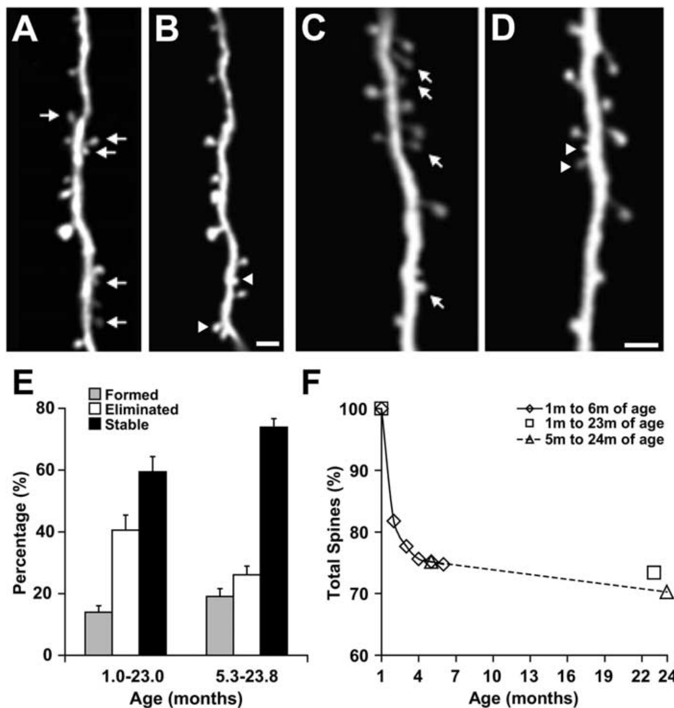

Figure 4. The Majority of Dendritic Spines in Adult Mice Persist over 18 Months

(A–D) Dendritic branches from two animals imaged 18 months apart show most spines remain at the same locations. (E) Percentage of spines eliminated, formed, and stable over 18–22 months. One group of mice was imaged between 1 and 23.0 ± 2.6 months of age. Another group was imaged between 5.3 ± 1.0 and 23.8 ± 2.6 months of age. (F) Percentage of total number of spines as a function of age from 1 to 24 months of age. The arrows indicate spines that are eliminated in the second view. The arrowheads indicate spines that are formed in the second view. Data are presented as mean ± SD. Scale bar, 2 μm.

1.2% of spines were eliminated and 4.3% ± 1.3% were formed in motor cortex over 2 weeks (Figures 3J, 3K, and 3O; 224 spines, 2 animals). Similar turnover rates were found in frontal and barrel cortices over 2 weeks (Figures 3H, 3I, and 3O; frontal: 5.3% ± 0.8% elimination and 4.5% ± 1.0% formation, 549 spines, 4 mice; Figure 3O; barrel: 5.2% ± 1.4% elimination and 4.9% ± 1.0% formation, 603 spines, 3 mice). Furthermore, over a 1 month interval, 5.0% ± 2.6% of spines were eliminated and 7.1% ± 0.8% were formed in motor cortex (352 spines, 3 mice), comparable to those in barrel and visual cortices (Figures 3L, 3M, and 3O; Grutzendler et al., 2002). Thus, spines showed very similar stability in all four cortical regions over either 2 or 4 weeks.

To better understand changes in spine dynamics as animals mature, spine dynamics were compared in barrel cortex from mice at 1, 2, 4, 5, or 6 month of age. Over a 1 month interval, spine elimination is 10.4% ± 1.7% for 2-month-old animals (340 spines, 3 mice), significantly lower than that for 1-month-old mice (Figure 3N, 23.4% ± 0.8%, 813 spines, 3 mice; $p < 0.01$) but higher than that for >4-month-old animals (Figure 3O, 6.0% ± 2.1%; 883 spines, 6 mice; $p < 0.01$). Furthermore, there is no significant difference in the rate of spine elimination among animals at 4, 5, and 6 months of age. Similar results were found in the visual cortex (Grutzendler et al., 2002). Thus, the rate of spine elimination gradually decreases between the first and fourth months of postnatal life.

Unlike the significant reduction in spine elimination, the percentage of spine formation over a 1 month interval was comparable in young adolescents and adults (Figures 3N and 3O; 1 month-old: 5.1% ± 1.4%; 2-month-old: 5.4% ± 1.5%; >4-month-old: 5.5% ± 2.8%). Based on the rates of elimination and formation over 1–6 months of age, we calculated that an ∼25% net loss of spines occurs from 1 to 4 months of age and that the number of spines remains relatively stable afterward (Figure 4F). Consistent with this in vivo time-lapse result, in fixed brain slices of the barrel cortex, we found an ∼36% reduction in spine density on the apical dendrites of layer 5 pyramidal cells from youth (44.5 ± 4.8 spine/100 μm, 446 spines, two 1-month-old mice) to adulthood (28.5 ± 3.5 spine/100 μm, 408 spines, two 5.5-month-old mice). Together, these findings indicate that a substantial number of spines are eliminated during adolescence before spines become stabilized in adulthood.

Majority of Adult Spines Are Maintained over Nearly the Entire Lifespan of an Animal

Assuming a stochastic turnover of spines at the rate of 5% per month, we estimated that ∼40% of spines would last over 18 months, almost the entire adult life span of the mice. To test this experimentally, we directly imaged the same dendrites over an interval of 18 months in adult barrel cortex. Remarkably, we found that 73.9% ± 2.8% of spines were still present while 19.1% ± 2.5% of spines were formed over 18.5 ± 2.4 months (Figure 4; 433 spines, 4 mice). The percentage of spines formed is significantly lower than that eliminated (26.1% ± 2.8%) ($p < 0.005$, paired t test), indicating a decrease in spine number over 18 months in adulthood. Consistent with this finding, we found that despite substantial spine loss during young adolescence, 59.5% ± 4.9% spines were stably maintained, with 14.0% ± 2.1% of spines formed between 1 and 23 months (231 spines, 22.0 ± 2.6 month interval, 3 mice;

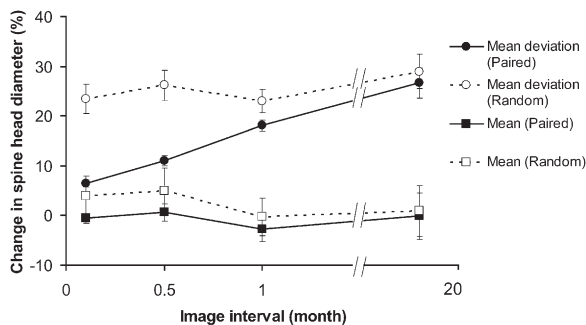

Figure 5. Spine Head Diameters Undergo Progressive Changes over Time

While the mean of the spine head diameter (relative to the adjacent shaft diameter) does not show a significant increase or decrease over various time intervals (3 days to 18 months, one-way ANOVA, $p > 0.8$) (solid squares), the mean deviation (the average of the absolute values of all the deviations) from the mean percent change in spine head diameter increases progressively over time (solid circles). Both the mean (open squares) and mean deviation (open circles) of random change in spine head diameter (randomly pairing different spines between two views) remain relatively constant over different intervals. The mean deviation corresponding to randomly paired spines is comparable to the paired mean deviation over 18 months ($p > 0.8$, t test), suggesting that extensive changes in spine morphology occur during this period. Data are presented as mean ± SEM.

Figures 4E and 4F). These data demonstrate that after a substantial spine loss during young adolescence, the majority of adult spines are highly stable and can last over the lifetime of an animal.

The measured adult spine stability, 74% over 18 months, is much higher than the calculated value (~40%) based on 5% spine turnover per month. Assuming that all the spines have the same turnover rate over 18 months, we calculated from the 18 month data that the average turnover rate of spines should be 1.6% per month, instead of the measured 5% per month. One possibility accounting for such a difference is that a small population of spines (up to 3.4%) may undergo more rapid turnover than the rest within a 1 month interval. To test this possibility, we imaged two adult animals three times, with a 1 month interval between two consecutive views. In 25 dendritic segments containing 161 spines in the initial view, seven spines were formed in the second view and two of them were eliminated in the third view. Thus, newly formed spines within the first month were eliminated at a much higher rate (2/7, 28.6%) within the next month than the entire spine population (6.0% ± 2.1%). However, this newly formed spine population (2/161, ~1.2%) does not appear to account for all the difference between the measured and calculated spine stability. This suggests that other factors are likely to be involved in the long-term stability of spines over 18 months (see Discussion).

Changes in Spine Morphology Occur Throughout Life

Previous studies have suggested that spine head diameter correlates well with the size of the postsynaptic density (Harris and Stevens, 1989). Changes in spine morphology could thus indicate alterations in synaptic strength. When the diameters of identified spines were compared over various time intervals, we did not observe a trend toward spine enlargement or reduction in both young and adult barrel cortex ($p > 0.1$; paired t test). Because imaging and animal conditions varied over time, we also measured the spine head diameter relative to the adjacent dendritic shaft diameter and calculated the percent change of this relative spine head diameter between views (see Experimental Procedures). Over different time intervals, we found that the mean of the percent change in spine head diameter is close to zero and is not significantly different from each other (Figure 5; one-way ANOVA, $p > 0.8$). However, we did observe that many spines underwent either increases or decreases in spine head diameter. In adult mice, with prolonged imaging intervals, there is a progressive increase in the mean deviation (average absolute deviation) from the mean percent change in spine head diameter (6.4% ± 0.7% over 3 days; 11.1% ± 1.0% over 2 weeks; 18.0% ± 1.1% over 4 weeks; 26.7% ± 3.1% over 18 months; 60 to 80 spines analyzed from three animals for each time interval; mean ± SEM) (Figure 5, solid circles). These observations suggest that changes in spine head diameter (either increase or decrease) accumulate with time. In addition, assuming that all spines undergo random changes in their morphology, we randomly paired different spines and found that the mean deviation from the mean percent change in spine head diameter ranged from 23.0% ± 2.4% to 29.0% ± 3.5% over various time intervals (Figure 5, open circles). These values are comparable to those of the observed percent change in spine head diameter over 18 months ($p > 0.6$, t test), suggesting an extensive fluctuation in spine morphology during this period. Furthermore, although the rate of spine turnover is high during young adolescence, the mean deviation over 2 weeks in young mice is similar to that in adults (12.0% ± 1.4% for young animals versus 11.1% ± 1.0% for adults; $p > 0.5$, t test). Thus, while the number and distribution of adult spines remain remarkably stable over many months, rapid changes in synaptic strength can occur throughout life and provide a useful means to modulate synaptic connectivity.

Discussion

By repeatedly imaging the cortex of living mice over time, we examined spine dynamics on the apical dendrites of layer 5 pyramidal neurons in different areas of the cerebral cortex. We found that in adults (>4 months old), spines are remarkably stable, exhibiting similar low rates of elimination and formation in barrel, primary motor, and frontal cortices. In adult barrel cortex, >70% of spines are present over an 18 month interval. Together with our previous studies in primary visual cortex (Grutzendler et al., 2002), these results indicate that a significant percentage of adult spines in the cerebral cortex can last throughout the life span of an animal. This cortical-wide spine stability could therefore provide a structural basis for lifelong information storage.

Our results also show that spine stability is achieved progressively as mice reach adulthood. In the first few postnatal months, the rate of spine elimination is significantly higher than that of formation, resulting in a concurrent net loss of spines in layer 5 pyramidal cells in different cortical regions. These observations are consistent with previous studies from fixed tissue showing that synaptic density in the mammalian cortex decreases substantially from infancy until puberty (e.g., Huttenlocher, 1990; Lubke and Albus, 1989; Markus and Petit, 1987; Rakic et al., 1986). Because similar phases of synapse development occur in diverse regions of the cerebral cortex in different species, it is possible that synapses of different cell types and cortical layers reach adult stability after undergoing a similar pruning process. Synapse loss during adolescence correlates with the development of many cortical functions and the malleability of the nervous system. Such a loss may therefore reflect experience-dependent remodeling of the developing brain as the animal adapts to its environment (e.g., Lichtman and Colman, 2000).

The degree of adult spine stability (~5% spine turnover per month) in our study differs substantially from that in a previous study, which showed that ~20% of spines turn over within 1 day and ~40% of spines turn over within a week in barrel cortex and led to the conclusion that spines are highly plastic in the adult brain (Trachtenberg et al., 2002). Recent studies from the same group indicated that the high spine turnover rate is partly due to the use of young adult mice (6–10 weeks of age) instead of mature adults (Holtmaat et al., 2005). Although this newer study showed that the majority of adult spines are stable over weeks, 21.4% ± 4.3% of total spines in 6-month-old mice are still considered transient and persist less than 4 days (Holtmaat et al., 2005). Such a high percentage of a transient population of spines, however, is not observed in our study (Figure 3O). Besides differences in animal ages, we believe that two other major factors contribute to the dramatically different spine stability observed in adult cortex (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005). The first factor is related to differences in imaging approaches used in these studies. In our study, spines were imaged only two or three times through a thinned intact skull, ~15 μm in thickness. Comparison of the same images taken from such a thinned skull and no-skull window showed that the thinned skull window does not prevent dendritic protrusions from being detected (Grutzendler et al., 2002; Figure S2). In other studies, spines were imaged through a cranial window that involves removal of ~5 mm in diameter of the skull, implanting 1.2% agarose and a coverslip (Trachtenberg et al., 2002) as well as adding a potent steroid, dexamehtasone, to minimize swelling at the surgical sites (Holtmaat et al., 2005). Images were taken multiple times on a daily basis and usually started 7–10 days after the craniotomy had been performed (Holtmaat et al., 2005; Trachtenberg et al., 2002). We believe that transcranial two-photon imaging of neuronal structures is a relatively straightforward and minimally invasive method, allowing repeated imaging over periods ranging from minutes to years. In contrast, the open skull method often causes bleeding from blood vasculature within the brain, inflammatory reactions, and neurite damage, which could lead to significant increase in spine turnover, particularly on superficial dendrites in layer 1 (Figures S2 and S3).

Another factor contributing to the difference in adult spine stability is that previous studies in barrel cortex did not distinguish filopodia from spines in the analysis of spine dynamics (Trachtenberg et al., 2002; Holtmaat et al., 2005). Our studies showed that filopodia are highly abundant in young animals, but are scarce (~2%–3%) in various adult cortical regions. Even if we include filopodia data in our spine quantification, we would find that only ~8% of all protrusions turn over per month in adulthood, significantly lower than 21.4% turnover over 4 days (Holtmaat et al., 2005) and ~40% turnover over 1 week (Trachtenberg et al., 2002). Therefore, we believe that a combination of factors such as the use of young animals, the invasiveness of the open skull technique, and inclusion of filopodia as spines must all contribute to the very high turnover rates of adult spines reported in previous studies in barrel cortex (Trachtenberg et al., 2002; Holtmaat et al., 2005). These factors may also lead to different results regarding the time period over which net spine loss occurs: while we observed a substantial reduction in spine number between 1 and 3 months of age in different cortical regions (Figures 3 and 4; Grutzendler et al., 2002), the other group reported that a net spine loss occurred from postnatal day 14 to 26 but not afterward in barrel cortex (Trachtenberg et al., 2002; Holtmaat et al., 2005).

Many lines of evidence indicate that filopodia and spines, although related, are structurally and functionally different entities. Filopodia are thin and long protrusions without a bulbous head. Unlike spines, filopodia contain little or no AMPA receptors that are important for synaptic transmission (Matsuzaki et al., 2001). The lifetime of filopodia in both young and adult cortex is on the order of hours, whereas spines have a lifetime of many months, further suggesting different molecular compositions between the two structures. Consistent with the view that filopodia serve as precursors of spines (Dailey and Smith, 1996; Fiala et al., 1998; Ziv and Smith, 1996), we found that the vast majority of filopodia undergo rapid turnover within hours but that only a few percent of them are converted to spines lasting 1–2 days. Because filopodia are fundamentally different from spines in structure and function, it is important to measure and quantify their dynamics separately with appropriate temporal resolutions. For example, because >50% of filopodia turn over within 4 hr in young adolescent barrel cortex, we estimate that the total number of filopodia eliminated and formed within 3 days is comparable to the total number of spines. Including filopodia as spines in the quantification of spine dynamics would suggest that at least 50% of spines turn over within 3 days. Such a turnover rate clearly does not represent the fate of the majority of spines. It is important to note that previous studies (Trachtenberg et al., 2002; Holtmaat et al., 2005) observed changes of many "transient spines" over 1 day but unfortunately did not examine their dynamics over

hours, therefore likely missing the true dynamic nature of these transient spines. In fact, the vast majority of such transient spines appear to be filopodia and have little interchange with persistent spine pools (Holtmaat et al., 2005). Labeling filopodia as transient spines and subsequently combining filopodia and spines together in data analysis and interpretation would generate confusion in understanding spine dynamics. Such a mixing plus the use of young animals and open skull imaging has led to a view that adult spines are highly dynamic (Trachtenberg et al., 2002). This mixing plus open skull imaging also lead to the suggestion that spines are more dynamic in barrel cortex than in visual cortex in adulthood (Holtmaat et al., 2005), whereas our data revealed strikingly similar spine dynamics in the two sensory cortices.

Our results show that the percentage of adult spines eliminated and formed slowly increases with time, but the majority of spines can last over the lifetime of the animal. It is interesting to note that spine turnover is not linearly proportional to imaging intervals (Figure 3O). For example, in adult barrel cortex, 2.1% ± 0.5% of spines were eliminated and 1.5% ± 0.9% were formed over 3 days. Over a 1 month interval, 6.0% ± 2.1% and 5.5% ± 2.9% of spines were eliminated and formed, respectively. Over 18 months, however, only 26.1% ± 2.8% of spines were eliminated and 19.1% ± 2.5% were formed. Several factors might lead to the nonlinear relationship between the rate of spine turnover and the imaging interval. First, it is possible that a certain population of spines is more prone to change than the rest. This pool might include, for example, spines that are newly formed from filopodia. Consistent with this, we found that 2%–3% of total protrusions are filopodia in adults. Although the vast majority of adult filopodia do not directly lead to spines lasting over 1–2 days, they may generate a small percentage of spines that turnover more quickly than the rest. Second, because filopodia are classified based on the length and the ratio of “head” to “neck” diameter, those in the process of retraction are morphologically indistinguishable from stubby spines. Thus, a small percentage of dynamic filopodia may be included as stubby spines in the quantification of spine dynamics. If so, the percentage of filopodia might be slightly higher and short-term spine dynamics might be lower than we have estimated. Future use of morphological criteria in combination with molecular marks (e.g., AMPA receptors) to distinguish filopodia from spines would be useful to address this issue. Third, anesthesia and slight rotation of dendritic structures between views may result in artifactual changes of a small percentage of spines, contributing to the dynamic pool of spines. Last, mice from 4 to 6 months of age were used for determining adult spine dynamics over a 1 month interval. It is conceivable that spines could become even more stable as adult animals age. Regardless of the factors contributing to the dynamic pool of spines, we estimated that this dynamic pool could be up to 3.4% of the total spine population, based on the observed spine dynamics over 1 month and 18 month intervals. If we assume that the rest (96.6%) of the spines have the same turnover rate and that 74% of them last over 18 months, the average lifetime of an individual adult spine would be 63 months, much longer than the entire lifespan of the mice.

While the vast majority of spines are stable in adulthood, spine morphology undergoes progressive changes both during development and adulthood. Because spine size correlates with synaptic strength, changes in spine morphology indicate that synaptic strength can be modified without synapse turnover (Harris and Stevens, 1989; Matsuzaki et al., 2004). Such alterations could be important in the rapid adult plasticity seen in short-term learning (Buonomano and Merzenich, 1998; Grossman et al., 2002; Matsuzaki et al., 2004; Yuste and Bonhoeffer, 2001). It is important to note that our results of remarkable adult spine stability were obtained from mice under laboratory housing conditions. Therefore, it remains to be examined to what degree spine dynamics (number and strength) could be altered by an enriched environment and learning process. Furthermore, it is unclear to what degree presynaptic structures share similar stability as postsynaptic spines. In vivo time-lapse imaging of axonal terminals in identified cells in the peripheral nervous system have revealed age-dependent stabilization of presynaptic terminals at neuromuscular junctions and in submandibular ganglia (Gan et al., 2003; Lichtman et al., 1987). Whether similar stabilization of presynaptic components occurs in the cerebral cortex remains to be determined.

Experimental Procedures

Transgenic Mice

Transgenic mice (H-line, Feng et al., 2000) were purchased from Jackson Laboratory, housed and bred in Skirball Institute’s animal facilities. All experiments were done in accordance with protocols approved by the institutional animal committee.

Surgical Procedure and In Vivo Imaging of Dendrites

Mice aged P14 to 23 months of both sexes were used in the experiments. The procedure of transcranial two-photon imaging was described previously (Grutzendler et al., 2002). Great precaution was taken not to deflect the skull downward against the brain surface or to break through the bone during the thinning process, as minor brain trauma or bleeding may potentially cause inflammation and disruption of neuronal structures (Figure S3A).

Data Quantification

Analysis of spine dynamics was performed similarly as described previously (Grutzendler et al., 2002; see also the Supplemental Data). For quantifying changes in spine morphology, we minimized the possibility of rotational artifacts by preselecting from 3D image stacks only spines parallel to the imaging plane in both views. Images with spine heads containing saturated pixels were excluded. Imaging software (NIH ImageJ) was used to measure the maximum diameter of the spine head and nearby dendritic shaft diameter. The edges of spines/shafts were detected with a Sobel detector algorithm (ImageJ). Because imaging and animal conditions varied over time, the ratio of the spine head diameter to the adjacent dendritic shaft diameter was used as the calibrated spine head diameter. Change in the spine head diameter was defined as the difference of the calibrated spine head diameter between two views. Random changes in spine head diameters were calculated by randomly pairing different spines over various time intervals. Similar conclusions in Figure 5 were reached when we used the directly measured spine head diameter rather than the calibrated diameter (data not shown).

Two-dimensional projections of 3D image stacks containing in-focus dendritic segments of interest were used for all figures. The total number of spines was pooled from dendritic segments of dif-

ferent animals. Data throughout the text is presented as mean ± SD unless otherwise noted.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/46/2/181/DC1/.

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