

Effects of Synaptic Activity on Dendritic Spine Motility of Developing Cortical Layer V Pyramidal Neurons

Serkan Oray¹, Ania Majewska^{1,2} and Mriganka Sur^{1,2}

¹Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA and ²Picower Center for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

It is increasingly clear that dendritic spines play an important role in compartmentalizing post-synaptic signals and that their dynamic morphological properties have functional consequences. Here, we examine this issue using two-photon microscopy to characterize spine motility on layer V pyramidal neurons in acute slices of the developing mouse cortex. In this system, all spine classes except filopodia become less dynamic as development proceeds. General manipulations of activity (TTX or KCl treatment) do not alter spine dynamics, although increased glutamatergic transmission (AMPA or NMDA treatment) stabilizes developing cortical spines. These effects on spine dynamics do not appear to be related to AMPA or NMDA receptor expression as assessed with immunolabeling, as there is no correlation between spine motility and AMPA (GluR1/2) or NMDA (NR1/NR2B) receptor subunit expression on a spine by spine basis. These results indicate that activity through glutamatergic synapses is important for regulating spine motility in the developing mouse cortex, and that the relative complement of receptors, while different across morphological classifications, cannot account for differences in dynamic structural changes in dendritic spines.

Keywords: AMPA, development, mouse, NMDA, two-photon

Introduction

Most excitatory synapses in the mammalian cortex terminate on dendritic spines (Palay, 1956; Gray, 1959). Spines are structural specializations which are morphologically diverse and are likely to be involved in calcium compartmentalization and regulation (for reviews, see Hering and Sheng, 2001; Bonhoeffer and Yuste, 2002). Interestingly, spines also exhibit dynamic changes in size and shape which have been documented *in vitro* (Fischer *et al.*, 1998, 2000; Dunaevsky *et al.*, 1999; Korkotian and Segal, 2001) and *in vivo* (Lendvai *et al.*, 2000; Majewska and Sur, 2003). The role of these structural fluctuations is still unknown, though they may indicate periods of structural and synaptic rearrangement (Oray *et al.*, 2004). Notably, several recent reports have also shown that potentiating stimuli lead to an increase in spine size (Matsuzaki *et al.*, 2004; Okamoto *et al.*, 2004) while depotentiating stimuli lead to a reduction in spine size (Zhou *et al.*, 2004). Spine dynamics may therefore represent steady state oscillations of potentiation and depotentiation, and can have functional consequences (Majewska *et al.*, 2000b). Spine dynamics have also been shown to respond to synaptic activity through AMPA and NMDA receptors as well as to manipulations of general neuronal excitability in dissociated culture (Fischer *et al.*, 2000; Korkotian and Segal, 2001). However, the same effects were not observed in slice culture (Dunaevsky *et al.*, 1999). Defining the relationship, if any, between synaptic activation and spine dynamics in a system where more network

circuitry is preserved is critical for understanding the link between spine structure and function.

In cortex, AMPA and NMDA receptor subunits are developmentally regulated such that NR1 expression decreases (Catalano *et al.*, 1997), NR2B subunits are replaced by NR2A subunits (Carmignoto and Vicini, 1992; Sheng *et al.*, 1994; Flint *et al.*, 1997) and GluR2 expression, which reduces calcium permeability, increases (Kumar *et al.*, 2002) as development proceeds. Additionally, immature synapses are thought to exist in a 'silent' configuration composed primarily of NMDA receptors (Durand *et al.*, 1996; Shi *et al.*, 1999) which later gain AMPA receptors. These developmental changes in glutamatergic receptors suggest a situation where young, motile spines express an immature complement of receptor subunits which are then altered and replaced as the synapse matures. However, since calcium transients in spines appear to stabilize their motility (Fischer *et al.*, 2000; Korkotian and Segal, 2001), these same immature receptors, known to contribute to elevated post-synaptic calcium, might contribute to spine stabilization.

To determine whether synaptic activity regulates cortical spine dynamics and whether spine dynamics are related to glutamate receptor expression, we used two-photon microscopy in acute slices to examine the apical dendrites of cortical layer V neurons during a period when synaptogenesis is occurring throughout cortex. We find that spine motility is developmentally regulated and, in agreement with previous slice studies (Dunaevsky *et al.*, 1999), is unaffected by manipulating the general excitatory state of the network with bath application of TTX or KCl. Bath application of AMPA or NMDA, however, induced a reduction in spine motility, showing that synaptic transmission is linked to morphological dynamics. Further, we performed immunolabeling for GluR1, GluR2, NR1 and NR2B subunits on slices previously imaged for motility. We find no correlation between receptor subunit expression and spine motility, though there are differences in expression between morphological classes of spines. These data show that spine motility in cortical spines is regulated by glutamatergic transmission, yet whole-spine expression of glutamate receptor subunits assayed by immunolabeling cannot explain the variability in spine motility.

Materials and Methods

Slice Preparation and Pharmacology

Slices were prepared from postnatal day 9 (p9) to p20 transgenic mice expressing green fluorescent protein (GFP; transgenic line M) in a subset of cortical layer V neurons (Feng *et al.*, 2000). All experiments were performed under protocols approved by MIT's Institutional Animal Care and Use Committee and conformed to NIH guidelines. Mice were deeply anesthetized with sodium pentobarbital (35 mg/kg, i.p.; Henry Schein

Inc., Indianapolis, IN) and the brain was removed and transferred to a Vibratome (model 1000; Ted Pella Inc., Redding, Ca) containing a cold (4°C) solution containing (in mM): NaH₂PO₄ (1), NaHCO₃ (25), KCl (3), MgSO₄ (2), dextrose (10), sucrose (252), CaCl₂ (2.5) and kynurenic acid (5). Slices were cut in the coronal plane at a thickness of 300 μm while in this solution and then transferred to a holding chamber containing room temperature artificial cerebrospinal fluid (ACSF) containing (in mM): NaH₂PO₄ (1), NaHCO₃ (25), KCl (3), MgSO₄ (2), dextrose (10), NaCl (126), and CaCl₂ (2.5). Slices were allowed to equilibrate for 1 h at room temperature and were then transferred to a submersion chamber and continuously perfused with ACSF. Multiple slices from each animal were typically examined on each recording day. Some slices were additionally treated with 1 μM tetrodotoxin (TTX; Sigma, St Louis, MO), 10–60 mM KCl (Sigma), 2 μM (*S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Sigma), 50 μM *N*-Methyl-D-aspartic acid, (NMDA; Sigma) or 1–2 μg/ml Cytochalasin-D (Sigma) in ACSF while in the submersion chamber.

Two-photon Imaging

Images were collected on a custom-built two-photon laser-scanning microscope (Majewska *et al.*, 2000a) consisting of a Fluoview confocal scanhead (Olympus, Melville, NY) and a Ti:S laser (Tsunami, Spectra-physics, Menlo Park, CA) pumped by a 10 W solid state source (Millenia, Spectra-physics). The Ti:S laser provided 100 fs pulses at 80 MHz at a wavelength of 920 nm and fluorescence was detected with a photo-multiplier tube (HC125-02, Hamamatsu, Japan) in whole field detection mode. Layer V pyramidal neurons expressing GFP were first identified under low power (10× air lens, 0.25 NA, Olympus) and individual dendrites were then identified at higher power (60× water immersion lens, 0.9 NA, IR2, Olympus) with full-field epifluorescence illumination. The 60× lens was used for subsequent identification of spiny dendrites under digital zoom (×5–8) using two-photon imaging. Stacks with images spaced 1 μm apart in the *z*-dimension were acquired every 2 or 3 min at room temperature using Fluoview (Olympus) software over a period of 1–1.5 h with a digital resolution of 800 × 600 or 1024 × 1024 pixels. In experiments involving bath application of pharmacological agents, *z*-stacks were acquired for a control period of 45 min, followed by a wash in period of 10–15 min where no images were collected, and a second *z*-stack acquisition period of 45 min.

Immunostaining

After two-photon imaging, slices were fixed in 4% paraformaldehyde and then transferred to 30% sucrose for cryoprotection. The 300 μm slices were then frozen and resectioned in a cryostat (Reichert-Jung, now Leica, Frigocut 2800) at 40 μm. Floating sections were blocked and permeabilized with 0.5% Triton X-100 for 30 min in normal sera, incubated with primary antibodies for 48 h at 4°C, and subsequently incubated with secondary antibodies for 2 h at room temperature. Sections were then mounted in buffer, coverslipped, and sealed. The following antibodies were used: mouse anti-NMDA receptor subunit 1 (NR1) (1:250, 2 μg/ml; PharMingen, San Diego, CA), rabbit anti-NMDA receptor subunit 2B (NR2B) (1:500, 2 μg/ml; Upstate Biotechnology, Lake Placid, NY), rabbit anti-glutamate receptor subunit 1 (GluR1) (1:67, 2.2 μg/ml; Upstate Biotechnology), mouse anti-glutamate receptor subunit 2 (GluR2) (1:500, 2 μg/ml; Chemicon, Temecula, CA), goat anti-mouse Alexa 568 (1:200, 10 μg/ml; Molecular Probes, Eugene, OR) and goat anti-rabbit Cy5 (1:200, 7.5 μg/ml; Chemicon, Temecula, CA).

Confocal Microscopy

Immunostained sections were examined with full-field epifluorescence illumination in order to identify the neurons which had previously been imaged with two-photon microscopy. Confocal scanning microscopy was performed on a Bio-Rad (Hercules, CA) MRC-1024ES confocal system mounted on a Zeiss (Oberkochen, Germany) AxioPlan microscope. All images were collected with a Zeiss Plan-Neofluar 40× oil-immersion objective with numerical aperture 1.4. Fluorescence images were obtained with a krypton-argon laser with standard lines at 488 nm (GFP), 568 nm (Alexa568), and 647 nm (Cy5) with standard filters. *Z*-stacks encompassing the same volumetric region that had previously been imaged with two-photon microscopy were gathered with a digital resolution of 1024 × 1024 pixels with digital zoom (×6–10) at 1 μm

increments. In order to maintain image registration and minimize bleedthrough, the three fluorescence images from the GFP and two secondary antibodies were collected sequentially with each increment of the focal plane.

Two-photon Image Analysis

Images collected with the two-photon microscope were exported to MATLAB (MathWorks Inc., Natick, MA) and processed using custom-written algorithms. The four-dimensional data sets (*x*, *y*, *z*, *t*) were reduced to three dimensions (*x*, *y*, *t*) by calculating two-dimensional maximum projections of each image stack. This method of analysis underestimates spine dynamics since movements in the *z*-direction are not analyzed, but has the advantage of improving image contrast. The image projections were then median-filtered to reduce shot noise, and aligned to the first time point using a cross-correlation analysis. Individual spines were characterized with three measurements: the length of the spine as a function of time (measured from dendrite to spine tip), the diameter of the spine neck (approximate) and the diameter of the spine head (measured at its widest point). Spine motility was defined as the mean change in spine length per unit time (μm/min), calculated by summing the total absolute change in spine length and dividing by the total experimental time (Lendvai *et al.*, 2000; Majewska and Sur, 2003; Oray *et al.*, 2004). This measurement, in combination with the cross-correlation compensation for image drift, has the advantage of being insensitive to small variations in image alignment. Spine morphologies (stubby, mushroom, or thin) and filopodia were automatically defined using the measured head diameter, neck diameter and length for the first and last time points of every experiment. Briefly, spines were defined as stubby if they had a low length to neck diameter ratio ($L/d_n \leq 2$), and spines with a large length to neck diameter ratio could be either thin ($d_h/d_n \leq 1.3$) or mushroom ($d_h/d_n \geq 1.3$) based on the ratio of their head diameter to neck diameter. Filopodia were identified as long (>4 μm) protrusions. These definitions were based on standard criteria (Harris *et al.*, 1992) and a graphical depiction of the relevant parameters can be seen for the entire population of spines in Figure 3A. Values are reported as mean ± SE. *P*-values for correlations were calculated from a linear regression analysis and the comparison of control and drug conditions were performed using a parametric paired *t*-test.

Confocal Image Analysis

Images collected with the confocal microscope were also exported to MATLAB. The three fluorescent image stacks, corresponding to the GFP-labeled structure of the dendrite and the fluorescence from the two secondary antibodies, were in perfect register and alignment; therefore it was possible to use the GFP signal to demarcate the *x*, *y* and *z* boundaries of each individual spine and to then look at only these regions in the volumetric immunostaining stacks. The immunostaining intensity for each subunit was then calculated by integrating over all of the pixels within the volume which defined a single spine. Since the expression of receptor subunits should be enriched in spines as compared with dendrites, it was possible to normalize the immunostaining intensity at each spine with the intensity in an adjacent region of dendrite in order to account for local variations in staining intensity and in order to pool relative expression values across multiple dendrites and neurons. As with the spine motility analysis, relative expression values are reported as mean ± SE and *P*-values were calculated with linear regression analysis.

Results

Spine Motility as a Function of Age

We examined the motility of dendritic protrusions on GFP-expressing cortical layer V pyramidal neurons using two-photon microscopy in an acute slice preparation (Fig. 1). Neurons from a variety of cortical regions (visual, somatosensory, retrosplenial and motor cortex) with dendritic arborizations in multiple laminar compartments were imaged between the first and third postnatal weeks, a period of significant synaptogenesis across and within the individual layers of the rodent cortex (Wise

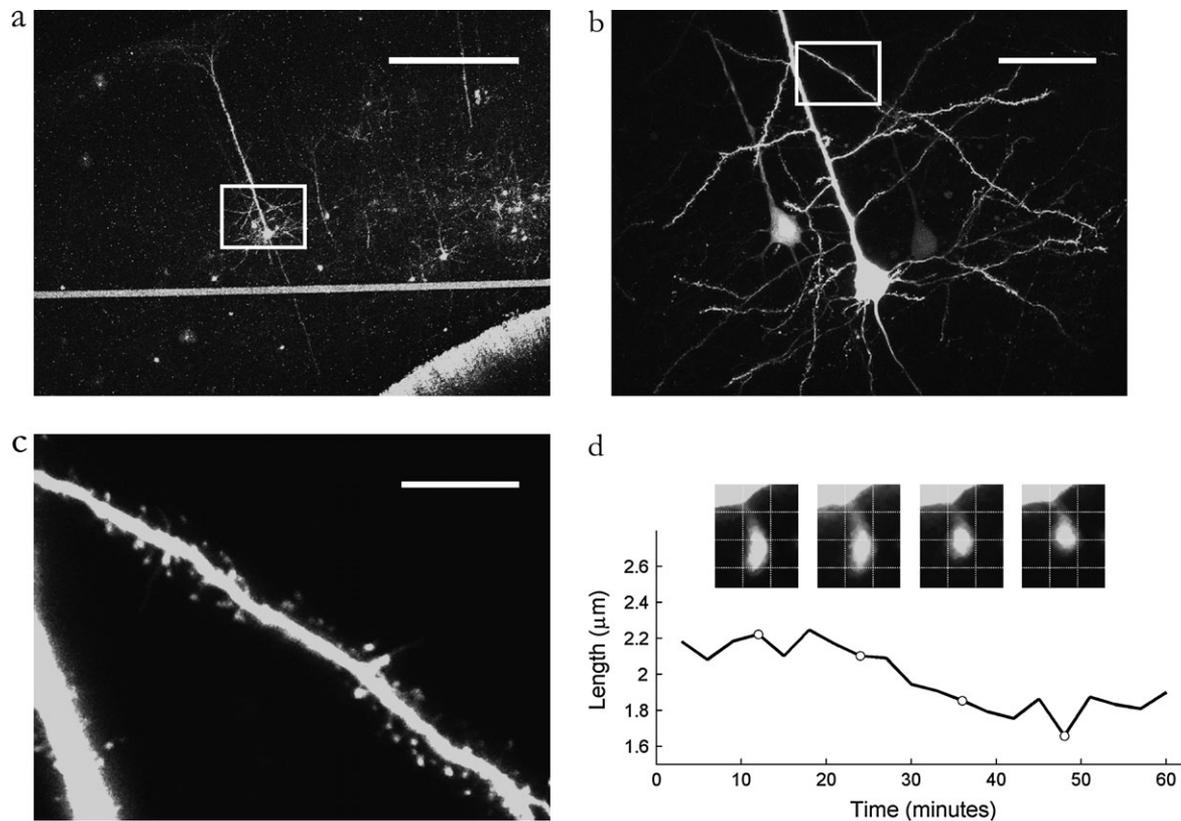


Figure 1. Acute slices from mice expressing GFP in a subset of their cortical layer V pyramidal neurons were imaged with two-photon microscopy at successively higher magnifications. (A) Multiple neurons express GFP in a $10\times$ field of view (scale bar, $400\ \mu\text{m}$). The long horizontal line is auto-fluorescence from a piece of nylon used to immobilize the slice. (B) At a magnification of $60\times$, it is easy to distinguish features of single cells including primary and secondary dendritic branches off the apical dendrite (scale bar, $50\ \mu\text{m}$). (C) By using the digital zoom feature of the laser scanning system, it is possible to clearly identify individual spines and to track their movement over time (scale bar, $10\ \mu\text{m}$). (D) The length of a single spine is measured over time. The inset images show the spine at each of the timepoints indicated by open circles in the plot. Spine motility is calculated from these length measurements as the mean change in spine length per unit of time (see Materials and Methods).

et al., 1979; Petit *et al.*, 1988; De Felipe *et al.*, 1997). As such, our results represent general properties of cortical layer V pyramidal neurons. The motility of individual protrusions was quantified over a period of 1–1.5 h by measuring the mean change in protrusion length as a function of time (see Materials and Methods). This was found to be the most consistent and simple metric to use for large numbers of spines and has been used successfully in previous studies (Lendvai *et al.*, 2000; Majewska and Sur, 2003; Oray *et al.*, 2004). The motility of apical dendritic spines on cortical layer V pyramids in acute slices decreased significantly with age (Fig. 2, $r^2 = 0.687$, $P < 0.001$, $n = 2388$ spines from 69 cells in 36 animals) as assessed by first order best fit linear regression. In order to facilitate a comparison with previously published spine motility values (Oray *et al.*, 2004), these data have also been resampled with longer time intervals and compared with spine motility from P28 animals (Fig. 2B, inset). Considering later developmental time points, spine motility is best fit with a second order exponential function with a rapid decline in motility at young ages and a much slower decline at older ages. This suggests that although spine motility declines rapidly during early development (p9–p14; see Konur and Yuste, 2004), possibly reflecting a very intense period of synaptogenesis, there is a slower decline towards a basal level of motility that is maintained into adulthood. In fact changes in spine morphology have been described in adult animals *in vivo* and have been postulated to be substrates for synaptic plasticity (Hayashi and Majewska, 2005; Zuo *et al.*, 2005). During the

imaging period, it was very rare to see spines appear and disappear. It is likely that spine turnover occurs over longer periods on the order of days and such changes might be difficult to capture in our acute slice preparation.

Additionally, we characterized spine morphologies by automatically categorizing protrusions into one of four morphological classes: stubby, mushroom or thin spines, and filopodia (Fig. 3). The decrease in spine motility seen across the entire population of dendritic spines was also seen within each of the three types of spines (thin $r^2 = 0.496$, $P < 0.05$, $n = 355$; mushroom $r^2 = 0.689$, $P < 0.001$, $n = 1364$; and stubby $r^2 = 0.653$, $P < 0.005$, $n = 669$), but not for filopodia ($r^2 = 0.214$, $P > 0.1$, $n = 214$) which exhibited high motility at all ages examined. These findings extend previous reports *in vivo* in the somatosensory system (Lendvai *et al.*, 2000) and in acute slices in the visual system (Konur and Yuste, 2004), and are consistent with observations of spine motility in dissociated culture (Korkotian and Segal, 2001) and slice culture (Dunaevsky *et al.*, 1999). As each of these previous reports has focused on different cell types and spine populations, the developmental decrease in spine motility is likely to reflect a general synaptic phenomenon in the cortex.

Spine Morphology as a Function of Age

In addition to characterizing spine motility over this two week period, we also considered developmental changes in spine

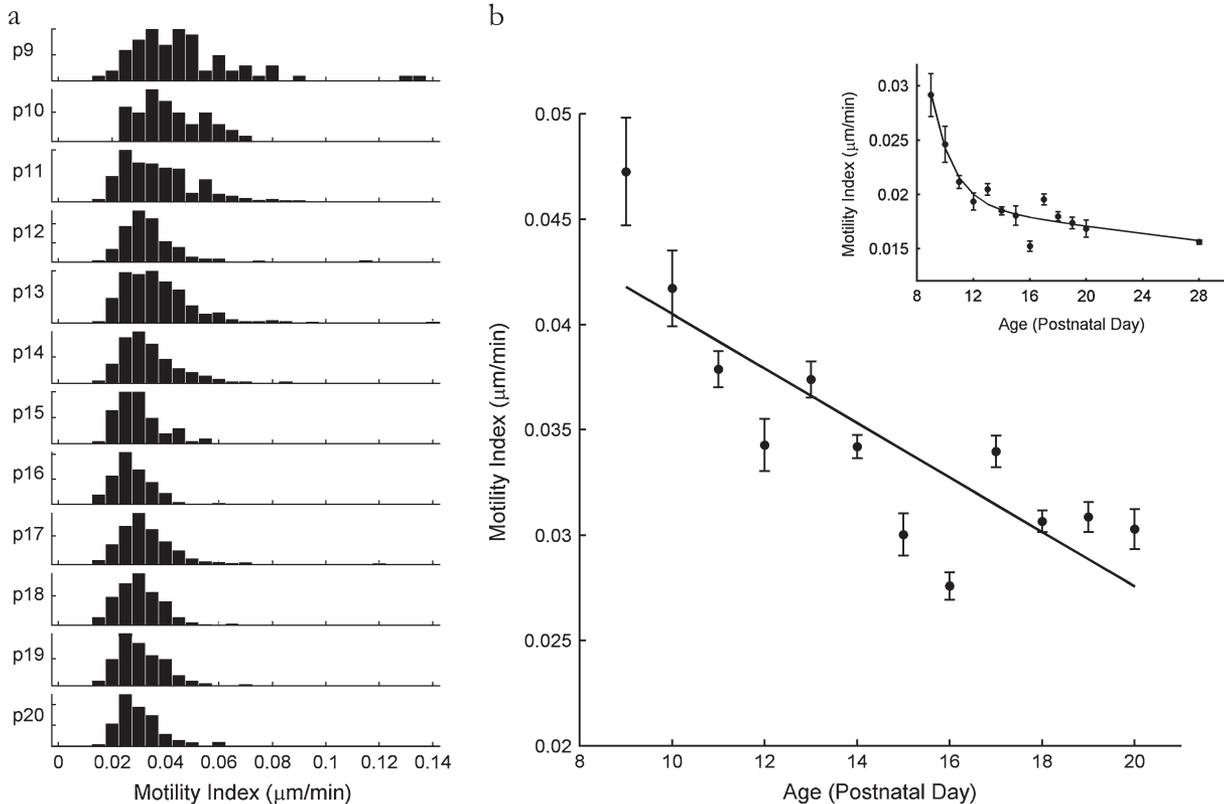


Figure 2. Spine motility (measured in $\mu\text{m}/\text{min}$ (see methods)) declines as a function of age. (A) Both the mean and variability of spine motility (excluding filopodia) decrease with age across the entire experimental population of spines. (B) A linear regression can be used to model the decrease in mean spine motility with age, though a second order exponential fit provides a better model when considering data from later timepoints (inset; data resampled at 6 min time intervals to match the analysis in Oray *et al.*, 2004).

morphology. It is generally considered that filopodia are an immature morphological protrusion and that mature spines tend towards mushroom and stubby shapes (Dailey and Smith, 1996; Ziv and Smith, 1996). Consistent with this idea, we observed an increase in the relative number of mushroom spines over this two week period (Fig. 3B, $r^2 = 0.569$, $P < 0.005$), rising from ~ 40 to 60% of the population. At the same time, the relative number of filopodia decreased significantly ($r^2 = 0.485$, $P < 0.05$) falling from $\sim 20\%$ to $< 5\%$ of the population. The relative number of thin and stubby spines remained constant over this period. These data suggest that filopodia are present in large numbers in young dendrites and are replaced by spines at older ages, dominated principally by mushroom spines.

Additionally, we considered whether the classification of spines changed over the course of our experiments, and whether this change was altered over the 2 week developmental period. It has previously been reported that the majority of spines maintain stable morphologies, though a relatively large number, $40\text{--}50\%$, shift their morphologies or become undetectable (Parnass *et al.*, 2000). We compared the morphologies of individual spines at the beginning and end of our experiments and observed that 28.6% (684 out of 2392) of spines changed their morphologies over the course of $45\text{--}60$ min (Table 1). This basal level of morphological shift can be primarily attributed to the conversion of thin spines into mushroom spines (6.79% of all spines) and the opposite conversion of mushroom spines into thin spines (8.13% of all spines). Conversion of thin to stubby spines (2.22%), stubby to thin (1.76%), stubby to

mushroom (3.73%) and mushroom to stubby (5.99%) proceeded at lower rates. There was no apparent age-dependence of the overall rate of morphological changes or on any of the individual types of morphological switching. This suggests that most of the developmental motility observed did not cause spines to change their morphological type and that there is no developmental trend in class switching.

Dependence of Spine Motility on Actin Dynamics

Dendritic spines are enriched in actin (Matus *et al.*, 1982) and their motility can be attributed to the polymerization and depolymerization of filamentous actin. Blockade of actin polymerization at the barbed end with Cytochalasin-D (Fischer *et al.*, 1998; Dunaevsky *et al.*, 1999; Star *et al.*, 2002) or sequestering monomeric actin with Latrunculin A (Fischer *et al.*, 1998; Korkotian and Segal, 2001) have been shown to inhibit spine motility. To confirm that the motility of spines on layer V pyramidal neurons was also actin-dependent, we imaged protrusions (both spines and filopodia) in acute cortical slices before and after application of $1\text{--}2 \mu\text{g}/\text{ml}$ Cytochalasin-D (Fig. 4A). The motility of young (p10) protrusions was significantly reduced by 43% (control $0.044 \pm 0.0021 \mu\text{m}/\text{min}$, cytochalasin $0.025 \pm 0.0009 \mu\text{m}/\text{min}$, $n = 37$, $P < 0.0001$). These data suggest that Cytochalasin-D reduces protrusion motility to $\sim 0.025 \mu\text{m}/\text{min}$ at this age, irrespective of the initial level of motility. We repeated this analysis after measuring the motility of old (p18) protrusions before and after Cytochalasin-D

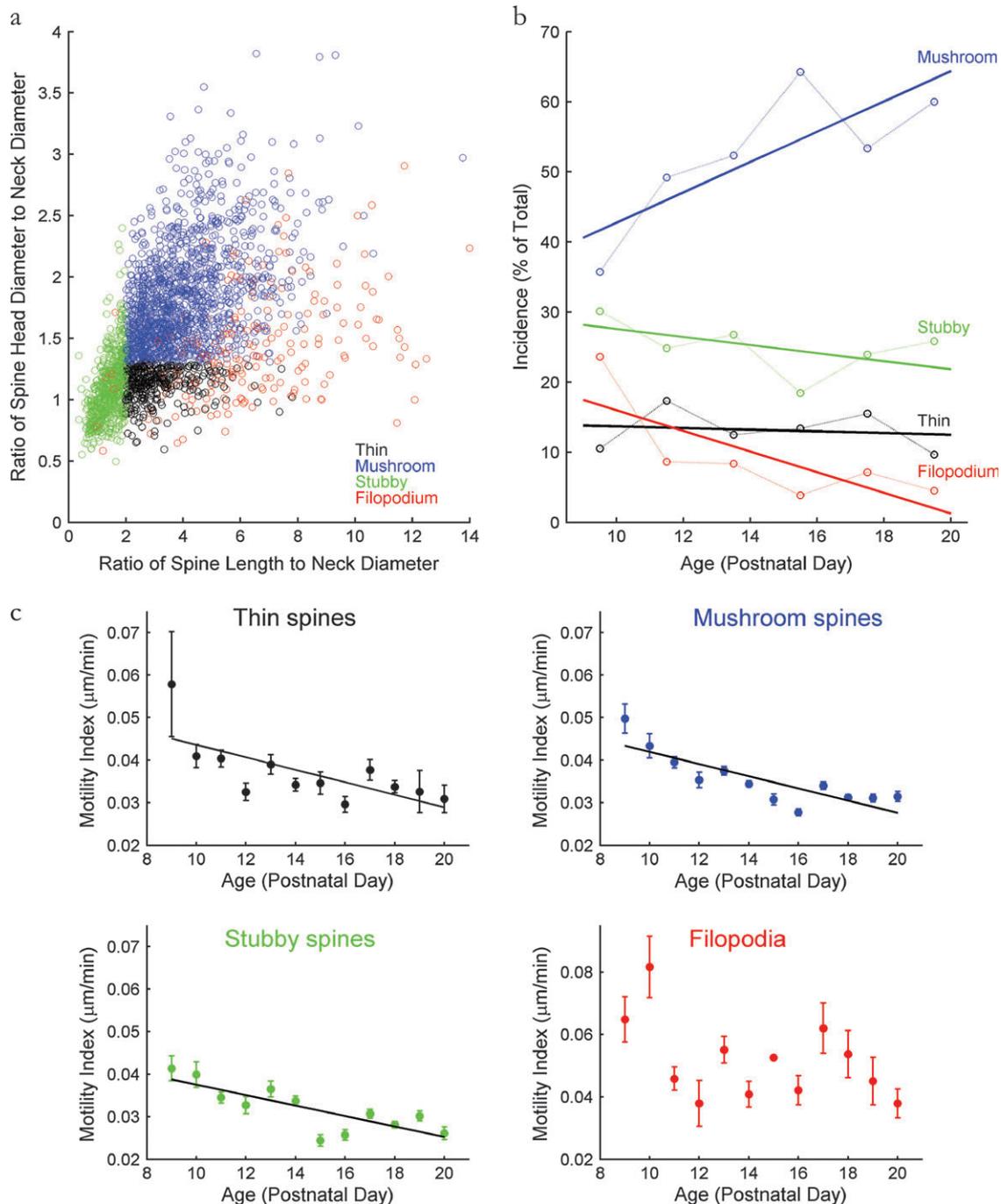


Figure 3. Spine morphology is developmentally regulated. (A) Spine morphology is automatically classified using standard criteria for spine length, neck diameter and head diameter. (B) Mushroom spines are the most common morphological classification and become more prevalent with development while the incidence of filopodia decreases. (C) The decrease in spine motility observed across the entire population of spines is also seen for each type of spine, while filopodia maintain a high level of motility throughout the developmental period.

treatment, and found that average motility was reduced by 41.6% (control $0.032 \pm 0.0031 \mu\text{m}/\text{min}$, cytochalasin $0.0187 \pm 0.0016 \mu\text{m}/\text{min}$, $n = 27$, $P < 0.0005$). This suggests that at this later age, Cytochalasin-D reduces protrusion motility to $\sim 0.020 \mu\text{m}/\text{min}$. These results were significant at both ages whether filopodia were or were not included in the analysis. Protrusion motility of $0.025 \mu\text{m}/\text{min}$ at p10 and $0.020 \mu\text{m}/\text{min}$ at p18 probably represents the average minimum motility when actin polymerization is blocked at filamentous actin barbed ends at these ages.

The difference in spine motility, both at baseline and after blockade, may also be indicative of different steady state depolymerization rates at different stages of development.

Dependence of Spine Motility on Activity

There are varying reports concerning the activity-dependence of spine motility. Increased extracellular KCl, which increases a neuron's excitability, has been shown to either inhibit motility

Table 1

Spine morphology transitions

	To thin	To mushroom	To stubby	Total spines
Thin	126 (37%)	161 (47%)	54 (16%)	342
Mushroom	193 (14%)	1075 (76%)	144 (10%)	1412
Stubby	43 (7%)	89 (14%)	504 (79%)	638

There is a low rate of change in spine morphology over short time intervals (45–60 min). Numbers represent the total number of transitions and percentages are calculated on the number of transitions in each morphological class. Numbers along the diagonal of the table represent spines with stable morphologies.

(Fischer *et al.*, 2000) or have no effect (Dunaevsky *et al.*, 1999). Specifically, in dissociated hippocampal cultures, increasing extracellular potassium to 6 mM was sufficient to inhibit spine motility (Fischer *et al.*, 2000), while others have been unable to see any effect in cultured slices in the range of 6–60 mM (Dunaevsky *et al.*, 1999). We assessed the effects of increasing extracellular potassium concentration on protrusion motility in young slices (p13) by raising the bath KCl concentration from 3 mM to 10 or 60 mM (Fig. 4B). In both cases, there was no effect of increased KCl on protrusion motility (control $0.046 \pm 0.0037 \mu\text{m}/\text{min}$, 10 mM KCl $0.044 \pm 0.0018 \mu\text{m}/\text{min}$,

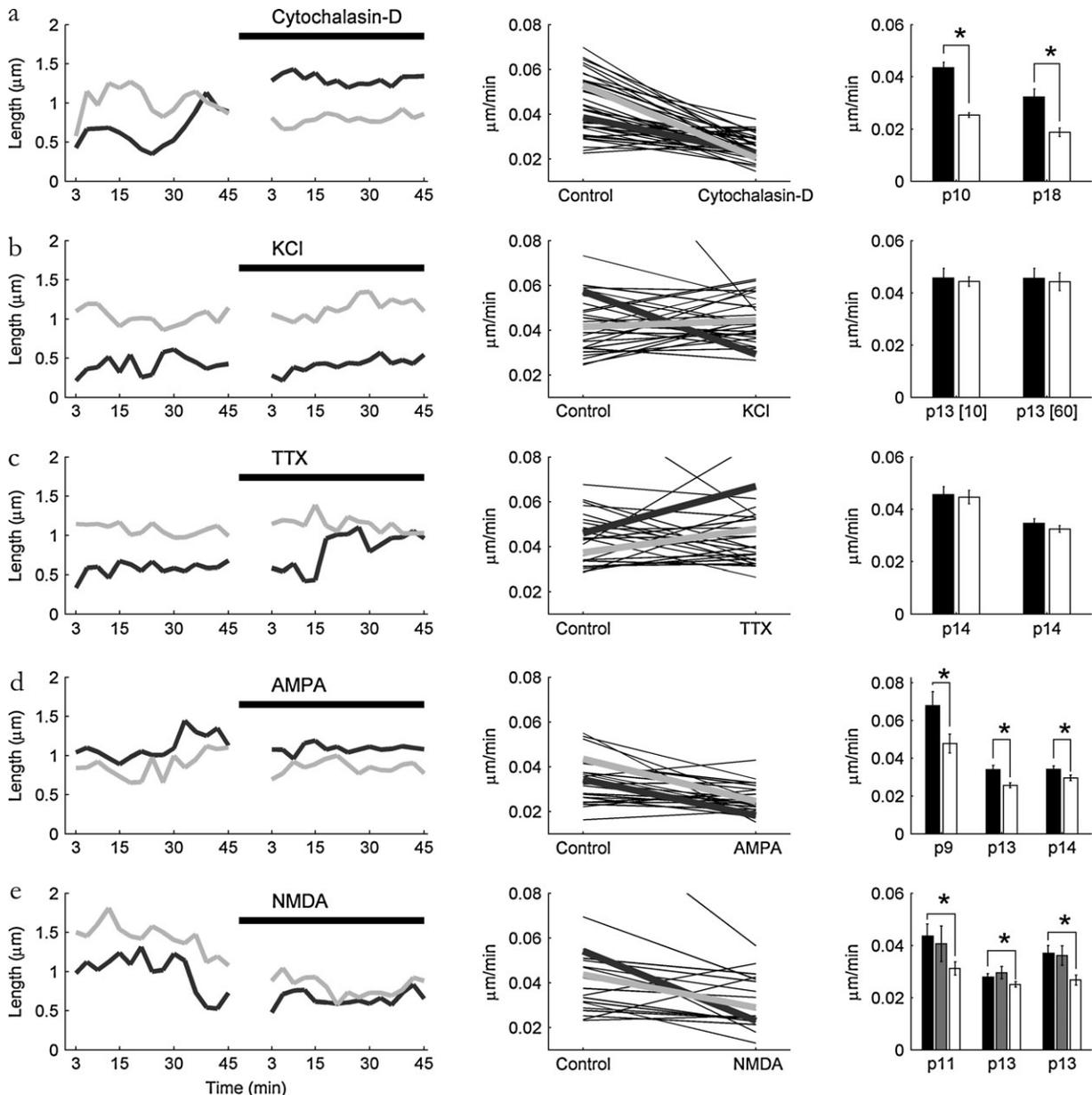


Figure 4. Protrusion motility is reduced by actin polymerization blockade and activation by AMPA or NMDA but is unaffected by generalized depolarization with KCl or action potential blockade with TTX. (A) 1 $\mu\text{g}/\text{ml}$ of Cytochalasin-D reduces spine motility significantly both in ‘young’ (p10) and ‘old’ (p18) spines. The leftmost panel shows two example spines (taken from the p10 experiment) which are very motile in the control period, and become much less motile after application of Cytochalasin-D. The middle panel shows the effect of Cytochalasin-D on all spines in this experiment and the two experiments are summarized in the right panel. (B) Depolarization with KCl (10 or 60 mM) at p13 has no effect on spine motility (example spines are taken from the 10 mM experiment). (C) Action-potential blockade with 1 μM TTX has no effect in two separate experiments at p14 (example spines are taken from the first p14 experiment). (D) 2 μM AMPA significantly reduces spine motility at p9, p13 and p14 (example spines are taken from the p13 experiment). (E) 50 μM NMDA significantly reduces spine motility at p11 and in two separate experiments at p13 (example spines are taken from the p11 experiment). Gray bars in the rightmost panel indicate spine motility in ACSF containing zero magnesium: spine motility is not significantly different from control ACSF. Asterisk, $P < 0.05$.

$n = 31$, $P > 0.5$; control $0.046 \pm 0.0038 \mu\text{m}/\text{min}$, 60 mM KCl $0.044 \pm 0.0034 \mu\text{m}/\text{min}$, $n = 29$, $P > 0.5$). The results were identical when filopodia were excluded from the analysis. This result confirms the previous results in cultured slice (Dunaevsky *et al.*, 1999) and suggests that there may be important differences in the efficacy of KCl on spine motility between slices and dissociated cultures.

Tetrodotoxin (TTX), a toxin which blocks sodium channels involved in action potential generation, has also been shown to be involved in controlling spine motility levels. In dissociated hippocampal culture, TTX has been shown to increase actin turnover (Star *et al.*, 2002) and to increase spine motility (Korkotian and Segal, 2001). We used 1 μM TTX to determine the effects of action potential blockade on protrusion motility in our acute slice preparation at p14 (Fig. 4C). In two experiments, we saw no effect of TTX on protrusion motility (experiment 1, control $0.046 \pm 0.0030 \mu\text{m}/\text{min}$, TTX $0.045 \pm 0.0026 \mu\text{m}/\text{min}$, $n = 27$, $P > 0.7$; experiment 2, control $0.035 \pm 0.0018 \mu\text{m}/\text{min}$, TTX $0.032 \pm 0.0013 \mu\text{m}/\text{min}$, $n = 53$, $P > 0.2$). As in the previous experiments, removing filopodia from the analysis gave the same result. As with the KCl treatment, there appears to be no effect of manipulating cell excitability on spine motility in our acute slice preparation.

Dependence of Spine Motility on Glutamatergic Activity

Since spines contain the post-synaptic elements of a synapse, their dynamics may influence synaptic efficacy and may in turn be influenced by synaptic transmission. In hippocampal cultures, spine dynamics were inhibited by activation of glutamatergic receptors while antagonists of the NMDA receptor had no effect on spine motility (Fischer *et al.*, 2000). Alternately, in cultured slices, glutamate, NMDA and CNQX, an antagonist of AMPA and kainate receptors, were found to have no effect on spine motility (Dunaevsky *et al.*, 1999). To investigate the effects of glutamatergic receptor activation on protrusion motility in our slice preparation, we bathed slices in either 2 μM AMPA (Fig. 4D) or 50 μM NMDA in zero magnesium ACSF (Fig. 4E). In the youngest protrusions (p9), 2 μM AMPA significantly reduced protrusion motility by 29.6% (control $0.068 \pm 0.0075 \mu\text{m}/\text{min}$, AMPA $0.048 \pm 0.0050 \mu\text{m}/\text{min}$, $n = 23$, $P < 0.05$). This effect was mediated by a strong reduction in the motility of filopodia as the difference disappeared after removing the filopodia from analysis (control $0.063 \pm 0.0084 \mu\text{m}/\text{min}$, AMPA $0.046 \pm 0.0065 \mu\text{m}/\text{min}$, $n = 16$, $P > 0.2$), though the general trend remained. At older ages, 2 μM AMPA also reduced protrusion motility by either 23.9% at p13 (control $0.034 \pm 0.0022 \mu\text{m}/\text{min}$, AMPA $0.026 \pm 0.0013 \mu\text{m}/\text{min}$, $n = 23$, $P < 0.05$) or 13.2% at p14 (control $0.034 \pm 0.0019 \mu\text{m}/\text{min}$, AMPA $0.030 \pm 0.0014 \mu\text{m}/\text{min}$, $n = 45$, $P < 0.05$). The results at p13 and p14 were nearly identical when filopodia were removed from analysis. Spine motility was also reduced by bath application of NMDA in the absence of external magnesium at p11 (control $0.044 \pm 0.0047 \mu\text{m}/\text{min}$, NMDA $0.031 \pm 0.0025 \mu\text{m}/\text{min}$, $n = 20$, $P < 0.005$) and in two separate experiments at p13 (experiment 1 control $0.028 \pm 0.0014 \mu\text{m}/\text{min}$, NMDA $0.025 \pm 0.0011 \mu\text{m}/\text{min}$, $n = 35$, $P < 0.05$; experiment 2 control $0.037 \pm 0.0030 \mu\text{m}/\text{min}$, NMDA $0.027 \pm 0.0019 \mu\text{m}/\text{min}$, $n = 34$, $P < 0.005$). These results were also identical whether or not filopodia were removed from analysis. For all three experiments with NMDA application, spine motility was briefly recorded while perfusing ACSF containing zero magnesium without NMDA over the slices. Spine motility in this condition was not

significantly different from spine motility in control ACSF in all three experiments ($P > 0.4$ in each experiment). These results, interestingly, confirm results in dissociated cultured neurons that protrusion motility is reduced by glutamatergic activity (Fischer *et al.*, 2000).

Glutamate Receptor Expression and Protrusion Motility

Since glutamatergic receptor expression is developmentally regulated in the cortex (Carmignoto and Vicini, 1992; Sheng *et al.*, 1994; Catalano *et al.*, 1997; Flint *et al.*, 1997; Kumar *et al.*, 2002) and since glutamatergic activation appears to be important for regulating dendritic protrusion motility, we were interested in examining whether glutamate receptor expression might correlate with spine motility. Based on the observation that AMPA and NMDA receptor subunits are developmentally regulated, young, motile spines might express an 'immature' set of receptor subunits and correlate positively with high levels of NR1 and NR2B, low levels of GluR2, and a low GluR1 to NR1 ratio, while older, more stable spines would have the inverse of these relationships. Alternately, since glutamatergic activation appears to stabilize spines and since post-synaptic calcium transients have been implicated in regulating spine motility in culture (Fischer *et al.*, 2000; Korkotian and Segal, 2001), receptor subunits which facilitate calcium influx, such as NR1, NR2B and the absence of GluR2, could contribute to protrusion stabilization and be negatively correlated with motility.

In order to correlate the expression of AMPA and NMDA receptors with protrusion motility, we fixed slices post-imaging and then double immunostained for pairwise combinations of NR1, NR2B, GluR1 or GluR2 receptor subunits. In low power images, these subunits were very highly expressed in the hippocampus and were expressed to a lesser degree in the cortex (cf. Rogers *et al.*, 1991). Using the characteristic dendritic arbors of the GFP-expressing neurons, we were able to identify, after immunostaining, the same spines which had previously been imaged with the two-photon microscope. The region of these spines was then re-imaged with high resolution confocal microscopy in order to assess the expression of the various receptor subunits. Numerical analysis was performed by first identifying the structure of the spine based on the structural GFP signal and then integrating the immunostaining signal over the entire volume of the spine. Figure 5 shows a typical experiment in which spines were first imaged with two-photon microscopy (Fig. 5A,B) and then immunostained for the AMPA receptor subunits GluR1 and GluR2 (Fig. 5C-G). To ensure that the immunostaining measured in this manner reflected glutamate receptor expression in spines rather than contaminating fluorescence from nearby structures, we plotted fluorescence profiles of glutamate staining vs. the GFP mask (Fig. 5G). The expression of glutamate receptors corresponded to the extent of spine labeling in the GFP channel and was high in the spine head, decayed at the edges of the spine, and was low in the dendritic portion of the GFP mask except for hotspots which might correspond to shaft synapses. We cannot rule out, however, that contamination from nearby structures contributes a small portion of the immunostaining signal in spines. This effect would be more pronounced in smaller spines and filopodia. In the experiment depicted in Figure 5, there was no correlation between GluR1 staining intensity or GluR2 staining intensity with protrusion motility (GluR1, $r^2 = 0.045$, $P > 0.3$; GluR2, $r^2 = 0.042$, $P > 0.3$, $n = 25$ from one cell).

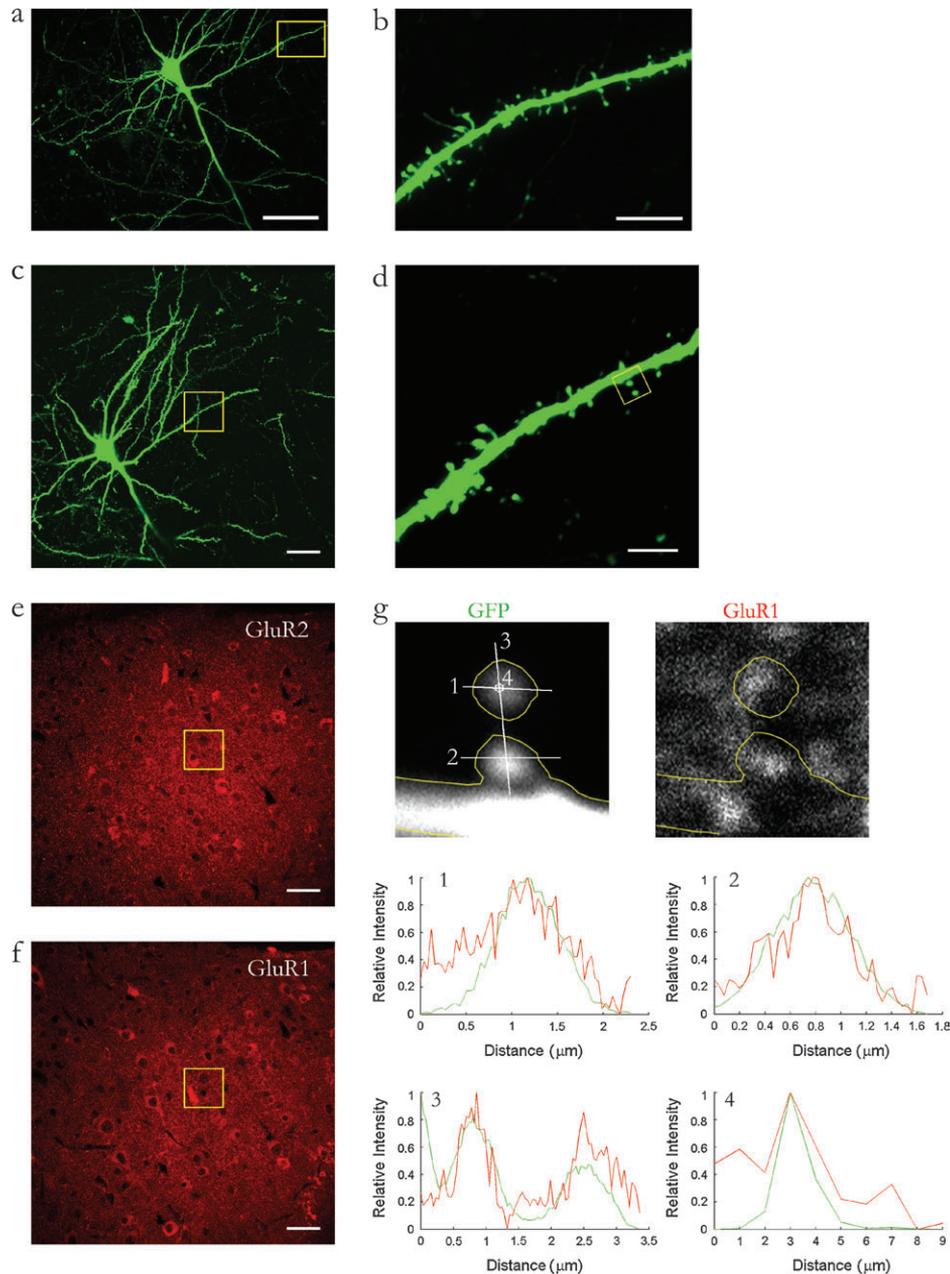


Figure 5. Determining the glutamate receptor composition of spines imaged in the acute slice. (A) Maximal z-projection of a neuron imaged with two-photon microscopy in an acute slice (scale bar, 50 μm). (B) shows the boxed region in (A) at higher magnification (scale bar, 10 μm) (C) The same neuron from (A) is re-imaged using confocal microscopy after it has undergone immunostaining for AMPA receptor subunits GluR1 and GluR2. This panel shows a maximal z projection of the GFP channel (scale bar, 50 μm). (D) A higher magnification view of the boxed region of (C) corresponding to spines imaged in the live preparation (B; scale bar, 10 μm). (E) Single z plane of the immunostaining for GluR2 (E) and GluR1 (F) of the region shown in (C); scale bars, 50 μm . G. Top panel shows two spines coming from the same dendrite (boxed region in D) in a single z-plane of an immunostained section (note that the spine neck of the topmost spine is not visible in this plane). Left panel shows the GFP-labeled dendrite and spines while the right panel shows the immunostaining for GluR1 (the outline of the GFP-labeled dendritic structures from the left panel is superimposed on the image). Notice that high levels of GluR1 staining restricted to both spines can be observed. Additionally, another punctum can be seen in the dendrite corresponding to a possible intracellular pool of receptor or a shaft synapse. Bottom panels show profiles of GFP (green) and GluR1 staining (red) fluorescence measured at the positions indicated in the left top panel. The fourth plot shows GFP and GluR1 fluorescence along the z-dimension at the intersection of lines 1 and 3. There is a good correspondence between the locations of GFP and GluR1 intensities at the spine boundaries indicating that puncta of GluR1 staining are restricted to spines. Areas of high GFP staining which correspond to the dendrite do not necessarily show high GluR1 staining (bottom panel for line 3; left side of graph).

This lack of correlation was consistent across the entire population of protrusions which were examined (Fig. 6A; GluR1, $r^2 = 0.0058$, $n = 168$, $P > 0.3$, 6 cells; GluR2, $r^2 = 0.0019$, $n = 80$, $P > 0.5$, three cells). Similarly, we found no correlation between NMDA receptor subunits and protrusion motility (NR1, $r^2 = 0.013$, $n = 223$, $P > 0.05$, 11 cells; NR2B, $r^2 = 0.001$, $n = 99$,

$P > 0.9$, four cells). These results suggest that AMPA and NMDA receptor expression is extremely variable on a spine to spine basis, and that this variability does not correlate with the observed motility of individual spines.

Since spine motility and glutamatergic receptor expression do not appear to be correlated, we asked whether simpler

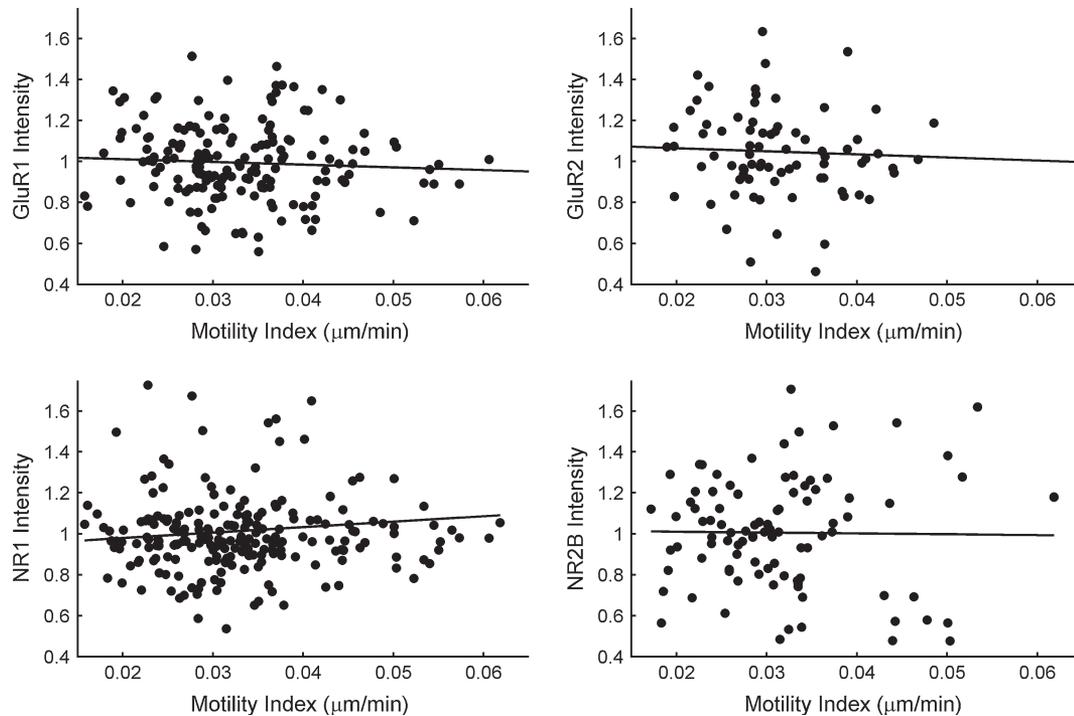


Figure 6. AMPA and NMDA receptor expression are insufficient to explain spine motility. Comparison of spine motility to GluR1, GluR2, NR1, and NR2 expression indicates that there is no correlation between receptor subunit immunostaining intensity and spine motility.

metrics, such as the size or morphology of a spine could be predictive of its receptor expression profile. There was no correlation between receptor expression and spine size (NR1, $r^2 = 0.0143$, $n = 216$, $P > 0.05$; NR2B, $r^2 = 0.0036$, $n = 92$, $P > 0.5$; GluR1, $r^2 = 0.0049$, $n = 161$, $P > 0.3$; GluR2, $r^2 = 0.0033$, $n = 74$, $P > 0.05$). Interestingly, mushroom spines showed slightly higher (~20%) expression of GluR1 ($P < 0.05$) than either thin spines or filopodia, and filopodia had significantly elevated levels of NR2B ($P < 0.05$ compared with thin, stubby spines and filopodia). Although it is difficult to determine whether the small differences in immunostaining intensity in small structures such as filopodia are indicative of significant differences in synaptic function, these results confirm a previous report that there are few AMPA receptors in filopodia or thin spines in comparison to high receptor expression in mushroom spines (Matsuzaki *et al.*, 2001). Taken, together, these results support the idea that filopodia may be immature protrusions which express relatively high levels of the 'young' NMDA receptor subunit NR2B and lack the AMPA receptor subunit GluR1, which is thought to convert 'silent' into functional synapses. The results also highlight that spine morphology cannot explain the variation in glutamatergic receptor expression in the thin, mushroom and stubby spine morphologies.

Glutamate Receptor Co-expression and Protrusion Motility

Since there was no obvious correlation between absolute receptor expression and protrusion motility, we wondered whether the important factor for motility might be the relative expression of different subunits with respect to one another. In general, due to nonlinearities in the immunostaining process, it is not possible to quantify the absolute number of receptors as a function of staining intensities, though it is possible to

measure their relative abundance qualitatively. In order to directly assess the effects of the coexpression of the various receptor subunits in our data set on spine motility, we examined the NR2B to NR1, GluR2 to GluR1, and NR1 to GluR1 ratios in relation to spine motility. None of these three ratios correlated with protrusion motility (NR2B/NR1, $r^2 = 0.008$, $n = 99$, $P > 0.3$; GluR2/GluR1, $r^2 < 0.006$, $n = 80$, $P > 0.4$; NR1/GluR1, $r^2 < 0.001$, $n = 88$, $P > 0.8$), indicating that the relative expression of receptor subunits, at least in the pool of receptors available in a spine, is not an important factor in regulating motility.

Additionally, we were interested in examining how well the components of AMPA and NMDA receptors correlated with the other subunits of the receptors. In fact, the correlation between NR1 and GluR1 expression in individual spines was very strong ($r^2 = 0.0762$, $n = 88$, $P < 0.01$), suggesting that, although there is a lot of variability, the relative expression level of these two receptor subunits was linked. The correlated expression of the NR1 and NR2B receptor subunits was also significant ($r^2 = 0.0409$, $n = 99$, $P < 0.05$), indicating that high levels of NR1 expression tended to also have high levels of NR2B expression. Interestingly, the expression of GluR1 and GluR2 receptor subunits was not significantly correlated ($r^2 = 0.018$, $n = 80$, $P > 0.2$), implying that GluR2 expression is not dependent on GluR1 expression. This supports the hypothesis that the presence of GluR1 and GluR2 receptor subunits are independently regulated at the level of the dendritic spine (Hayashi *et al.*, 2000; Shi *et al.*, 2001; Barria and Malinow, 2002).

Discussion

In order to assess whether synaptic activation influenced the structural dynamics of dendritic spines, we have examined spine motility on apical dendrites of layer V pyramidal neurons in an acute slice preparation. Our results help to resolve an

important debate regarding the importance of synaptic activity on dendritic structural dynamics by demonstrating, in a preparation with significant intact circuitry, a reduction in spine motility by glutamatergic activation of AMPA or NMDA receptors. This effect had previously been shown in dissociated culture (Fischer *et al.*, 2000), but was not observed in cultured slice preparations (Dunaevsky *et al.*, 1999). The stabilizing effect of synaptic activation on spines is in contrast to generalized depolarization with KCl or blockade of action potentials with TTX which had no effect on spine dynamics. These results also suggest an apparent disparity between experiments in dissociated culture systems (Fischer *et al.*, 2000; Korkotian and Segal, 2001) which find a strong effect of KCl and TTX on spine motility and those in cultured slice which find no effect. It is unclear why this should be so, though the difference could lie in the sparse connectivity of low-density cultures as opposed to the tissue-specific structure of connections present in slice preparations, or in the use of GFP-actin (as used in the culture experiments) to visualize spines rather than the spines themselves. These results argue strongly for the hypothesis that glutamatergic activity provides a regulatory mechanism for spine motility in intact systems and that this relationship is likely to be preserved *in vivo*.

Other experiments have also explored the importance of synaptic activity for regulating dendritic spines. Notably, local potentiation or depotentiation of synapses can bi-directionally alter spine size (Matsuzaki *et al.*, 2004; Okamoto *et al.*, 2004; Zhou *et al.*, 2004). Also, studies of synaptic activation with focal stimulation in hippocampal slice cultures have shown that filopodial outgrowth can occur within 30 min of potentiation (Maletic-Savatic *et al.*, 1999) or lead to the emergence of new spines over the course of several hours (Engert and Bonhoeffer, 1999). In these studies, it is not clear that spine motility and protrusion outgrowth are synonymous, as one can imagine a scenario where a local potentiating signal induces the outgrowth of a spine and then serves to stabilize the spine once it has appeared. Specific activation of AMPA receptors via spontaneous glutamate release also appears to be necessary for maintaining spine morphology and density in hippocampal slice cultures over a period of days (McKinney *et al.*, 1999). Interestingly, this study also found no effect of action potential blockade via TTX application in altering spine density or morphology profiles, suggesting that the maintenance is specific to AMPA receptor activation. This spine maintenance may be similar to spine dynamics as both are likely to be integrative processes where spines with inadequate or overabundant activation over long periods may alter their morphological and dynamic properties in response to their specific history of activation. The implication of these experiments is that synaptic activation is important for both the formation of new spines and presumably new synapses, as well as for altering spine morphology and structural dynamics, as we demonstrate.

One potentially important experimental paradigm which may resolve the role of spine motility in intact systems is the analysis of spines *in vivo*. In some areas of the cortex, spines and synapses are constantly being created and removed over long time scales (Trachtenberg *et al.*, 2002; but see Grutzendler *et al.*, 2002) in the intact animal, and in one study, spine motility was dramatically reduced in the somatosensory barrel cortex following whisker trimming (Lendvai *et al.*, 2000). This reduction in spine motility, at first, seems counterintuitive, given that reduced activity tends to increase spine motility in

dissociated cultures (Korkotian and Segal, 2001; Star *et al.*, 2002). Recent work in our laboratory suggests that the difference between the *in vitro* and *in vivo* work might also be an effect specific to the somatosensory system, as visual deprivation experiments results in increased spine motility *in vivo* during the critical period (Majewska and Sur, 2003). These findings have been extended by the observation that monocular deprivation elevates spine motility specifically in the binocular region of the visual cortex (Oray *et al.*, 2004). This process is dependent on the degradation of the extracellular matrix, suggesting a two-step model for structural plasticity where the adhesive matrix is first 'loosened' and subsequently the relative levels of activity through deprived and non-deprived inputs modulate spine dynamics. Further experiments are still needed to determine whether the relationship between activity-dependent mechanisms that regulate spine movement might also shape the precise formation and loss of synaptic connections.

Developmental Effects on Spine Motility

In the mouse cortex, synaptogenesis occurs at an accelerated rate for the first two postnatal weeks, but is ongoing until ~p32 (De Felipe *et al.*, 1997) and has been shown to occur concurrently across the cortex and throughout the cortical layers (Wise *et al.*, 1979; Juraska, 1982; Blue and Parnavelas, 1983; Petit *et al.*, 1988). During this period of elevated synaptogenesis in cortex, we find that spine motility on layer V pyramidal neurons throughout the cortex is actin-dependent and decreases for all spine types except filopodia, which remain highly motile but become less frequent in number as development progresses. These findings significantly extend a previous report of spine motility *in vivo* in layer II/III pyramidal cells in the somatosensory system (Lendvai *et al.*, 2000) and layer II/III cells in the visual system *in vitro* (Konur and Yuste, 2004). These findings also corroborate results from cerebellar, cortical, and hippocampal slice cultures (Dunaevsky *et al.*, 1999) and dissociated cell culture experiments (Korkotian and Segal, 2001) which found a decrease in spine motility with days *in vitro*, suggesting that the developmental decrease in spine motility is a common property of all morphological classes of spines (but not filopodia) as well as a general property of developing, spiny pyramidal neurons.

Glutamate Receptor Subunit Expression and Spine Motility

Having observed a strong influence of synaptic glutamate receptor activation on spine motility, we further examined the expression of glutamate receptor subunits on individual spines. Initially, we expected that spine motility would be either correlated with the developmental expression of AMPA and NMDA receptors, or with the ability to flux calcium ions which could then stabilize the cytoskeletal network. However, in our population of spines, the relative expression patterns of NR1, NR2B, GluR1, GluR2 or the ratios of the glutamatergic subunits did not correlate with the motilities of individual spines.

Since spine motility decreases with age, it is tempting to assume that the variations in motility at any point in development reflect the relative ages of individual spines, such that 'young' spines are more motile and 'old' spines are less motile. However, in cortex, the developmentally regulated change in the relative expression of glutamatergic receptor

subunits (Carmignoto and Vicini, 1992; Sheng *et al.*, 1994; Catalano *et al.*, 1997; Flint *et al.*, 1997; Kumar *et al.*, 2002) is superimposed on the reduction in spine dynamics. Our data suggest that these two processes are essentially independent and the subunit expression is insufficient for predicting spine motility. Further, subunits which might enhance the flux of calcium and potentially stabilize the cytoskeleton, such as the NR2B subunit, are present in abundance at young ages when spines are most motile and, in our data, on filopodia, suggesting that calcium cannot be the only determinant of a spine's dynamic profile. Rather, it seems likely that multiple, possibly homeostatic, developmental events shape both a spine's ability to respond to an influx of calcium and the complement of receptor subunits which determine its ability to respond to synaptic stimulation.

Finally, there is the consideration of whether immunostaining provides sufficient resolution to accurately detect receptor expression in individual spines and the functional distribution of subunits in the synapse. Certainly, immunostaining for receptors in a tissue section (Hohnke *et al.*, 2000) is more complicated than in cell culture (Shi *et al.*, 1999) and reflects both membranous and cytoplasmic receptor expression. However, there is evidence that the cytoplasmic receptor expression is proportional to expression at synapses (Mammen *et al.*, 1997; Rubio and Wenthold, 1999). Further, in experiments where exogenous AMPA receptor subunits were overexpressed in hippocampal neurons, once the subunits were driven into spines by mechanisms involving their cytoplasmic tails, their expression at synapses was detectable with electrophysiological recordings (Shi *et al.*, 1999; Hayashi *et al.*, 2000; Piccini and Malinow, 2002). These findings suggest that assaying endogenous receptor subunit expression in individual spines might be sufficient to estimate the amount of functional subunits incorporated at the synapse. Taken together, our findings suggest that spine dynamics are intimately related to glutamatergic synaptic activity, but that the expression pattern of glutamatergic receptor subunits appears to be insufficient to explain spine motility either from a developmental or cytoskeletal stabilization perspective.

Notes

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Address correspondence to Mriganka Sur, Massachusetts Institute of Technology, E25-235, 45 Carleton Street, Cambridge, MA 02139, USA. e-mail: msur@mit.edu.

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