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The role of glutamate in the morphological and physiological development of dendritic spines

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Abstract

Dendritic spines form the postsynaptic half of the synapse but how they form during CNS development remains uncertain, as are the factors that promote their morphological and physiological maturation. One hypothesis posits that filopodia, long motile dendritic processes that are present prior to spine formation, are the precursors to spines. Another hypothesis posits that they form directly from the dendritic shaft. We used microphotolysis of caged glutamate to stimulate individual dendritic processes in young hippocampal slice cultures while recording their morphological and physiological responses. We observed that brief trains of stimuli delivered to immature processes triggered morphological changes within minutes that resulted in a more mature, spine-like appearance in about half of experiments, such as decreases in spine neck length, increases in spine head width. We also observed that glutamate-induced inward currents elicited from immature processes were mostly or entirely mediated by NMDARs, whereas responses in those processes with a more mature morphology, regardless of actual developmental age, were mediated by both AMPARs and NMDARs. Consistent with this observation, glutamate-induced morphological changes were largely, but not entirely, prevented by blocking NMDARs. Our observations thus favor a model in which filopodia in the developing nervous system sense and respond to release of glutamate from developing axons, resulting in physiological and morphological maturation.

Keywords

glutamate; hippocampus; dendritic spines; dendritic filopodia

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HAM and SMT designed the experiments. HAM performed the experiments and HAM and DP analyzed the data. JPYK provided reagents. HAM drafted the manuscript, which all authors edited and approved.

INTRODUCTION

Dendritic spines form the postsynaptic half of the majority of excitatory synapses in the adult brain (Harris and Kater, 1994; Nimchinsky et al., 2002; Bourne and Harris, 2008; Kasai et al., 2010). Dendritic spines display a variety of shapes. Large, mushroom-shaped spines are the most stable category of spines and are most commonly observed at later stages of development and adulthood (Bourne and Harris, 2008; Knott et al., 2006). During early development, however, dendrites display primarily long, thin, highly motile, actin-rich processes, called filopodia, that lack the bulbous head characteristic of spines (Dailey & Smith, 1996; Ziv & Smith, 1996; Fiala et al., 1998). Even in the adult animal, transient, thin protrusions have been observed. Their stability increases with the acquisition of a mushroom-shaped morphology and the formation of a synapse (Knott et al., 2006). The processes by which spines develop and mature morphologically are incompletely understood, as is their physiological maturation. These processes are important because developmental delays in synapse maturation are a hallmark of several diseases of cognitive function (Penzes et al., 2011).

One hypothesis is that dendrites extend filopodia in order to contact potential presynaptic axons in their vicinity and initiate the formation of a synapse (Morest, 1969). Filopodia are thus precursors that later mature into spines. Imaging of dendrites in CA1 pyramidal cells in cultured hippocampal slices has revealed that filopodia are transiently protruded and retracted until chance contact with a nearby axon apparently triggers the accumulation of the pre- and postsynaptic molecular components (Ziv and Smith, 1996; Friedman et al., 2000), leading to ultrastructurally identifiable filopodial synapses (Fiala et al., 1998). An alternative hypothesis is that spines emerge directly from the dendritic shaft when there is direct contact between a growing axon and the dendrite.

Existing data on this process are conflicting. Some evidence (Ziv and Smith, 1996; Maletic-Savatic et al., 1999) suggests that filopodia become spines, whereas others have observed the direct protrusion of spines in the absence of preceding filopodia (Korkotian and Segal, 1999). Kwon and Sabatini (2011) have recently reported that tetanic stimulation of dendritic shafts with caged glutamate leads to the rapid outgrowth of spines with a mature morphology, lending support to the second hypothesis and raising questions about the role of filopodia in spine development.

Functionally, the size of the spine head in adults is tightly correlated with the number of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-preferring receptors (AMPA receptors) at the PSD (Takumi et al., 1999; Tanaka et al., 2005; Matsuzaki et al., 2001; Zito et al., 2009). Immature excitatory synapses, in contrast, contain many N-methyl-D-aspartate-preferring receptors (NMDARs), but few or no AMPARs (Isaac et al., 1995; Liao et al., 1995; Montgomery et al., 2001). With low frequencies of presynaptic glutamate release, such synapses are electrically silent at resting membrane potentials. The morphological correlate of silent synapses is unknown.

We used focal photolysis of caged glutamate to test the hypothesized role of immature protrusions in the genesis of mature dendritic spine morphology and asked whether

activation of the glutamate receptors present at these structures promotes the transformation of immature protrusions into a more mature morphology.

MATERIALS AND METHODS

All procedures used in the performance of this study were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee, in accordance with NIH guidelines for the care and use of animals.

Hippocampal slice cultures

Hippocampal slices were prepared from P0–7 rat pups or from P0–P4 mice expressing GFP under the control of the Thy1 promoter in subsets of pyramidal cells (line GFP-m; Feng et al., 2000) and cultured using the roller-tube technique (Gähwiler et al., 1998). In brief, slices were attached to glass coverslips in a clot of chicken plasma supplemented with fibrin and placed in a slowly rotating roller drum in a dry air incubator at 35°C. Cultures were allowed to mature for 3–21 days *in vitro*. We will use the term postnatal age to mean the sum of the postnatal (P) age of the animal in days at the time the tissue was explanted plus the number of days the cultures were maintained *in vitro* (DIV). CA1 cells in cultures from these mice constitutively express GFP and have well labeled dendritic processes.

Biolistic transfection

Cells in rat slice cultures were transfected with GFP (McAllister, 2000). Gold pellets (1.0 μm diameter) were coated with spermidine and then placed in a solution of 25 $\mu\text{g}/\mu\text{l}$ DNA, which was precipitated onto the pellets by the addition of CaCl_2 . Pellets were propelled into the slices using a Bio-Rad gene gun at a distance of 2 cm from the slice culture with a pressure of 200 psi. After transfection, slices were returned to roller tubes and placed in the incubator for 2–8 days.

Microphotolysis of caged glutamate

Stimulation of individual dendritic processes was performed using a solid-state diode pulsed laser (DPSS Lasers, output 1 W) fitted with UV optics to photolyze 1 mM *N*-(6-nitro-7-coumarylmethyl)-L-glutamate (Ncm-glutamate), a caged compound that generates free glutamate with rapid photorelease kinetics (<50 ns) in response to UV light (Cai et al., 2004). The laser beam was delivered through a 25 μm quartz fiber with the proximal end focused in a conjugate focal plane via relay lenses. The UV spot diameter had a full width at half maximum amplitude of approximately 1 μm (Bagal et al., 2005). Dendrites of GFP expressing or dye filled cells were visualized using wide field microscopy and a conventional mercury light source using a 60 $\times$ water immersion objective (0.8 na). The spot was then positioned at the tip of a postsynaptic protrusion to allow the rapid stimulation of postsynaptic receptors. The duration of the photostimulus pulse was determined by gating the acousto-optical Q-switch.

Electrophysiology

Cultures were placed in a 2 ml recording chamber with extracellular saline containing (in mM) 137 NaCl, 2.8 KCl, 2 CaCl_2 , 0 MgCl_2 , 11.6 NaHCO_3 , 2 HEPES, 0.4 NaH_2PO_4 , 0.02

phenol red, and 5.6 glucose, tetrodotoxin (TTX, 1 μ M) and picrotoxin (40 μ M). Whole-cell patch-clamp recordings were made from CA1 pyramidal cell somata. Patch pipettes (5–10 M Ω) were filled with 90 mM CsCH₃SO₃, 50 mM CsCl, 0.4 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 1 mM MgCl₂, 0.2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), titrated to pH 7.3 with 1 M CsOH. The pipette solution also contained 0.1 mM Alexa 568 for visualization and targeting of dendritic processes. D-amino-5-phosphonovaleric acid (AP5, 80 μ M) was used to isolate the AMPAR-mediated current component of the photolytic responses. The current persisting in AP5 was presumed to be mediated entirely by AMPARs, and was digitally subtracted from the control current to isolate the NMDAR-mediated current using Clampex software (Molecular Devices). The AMPA:NMDA ratio was calculated by dividing the peak AMPAR current by the peak NMDAR current after subtraction. When AP5 was not applied, the AMPA:NMDA ratio was calculated by measuring the peak amplitude of the response within 20 ms after the uncaging stimulus (AMPA-mediated response) and the average of the current between 30 and 50 ms after the uncaging stimulus (NMDAR-mediated response).

Morphological analyses

Images of living, fluorescently-labeled CA1 pyramidal cell dendrites were taken with a Nikon fixed stage microscope using a 60 $\times$, 0.8 NA objective and a Hamamatsu CCD camera (Orca ER; effective pixel size = 0.012 μ m²). Images were acquired and analyzed using C-imaging software (Compix, Hamamatsu Inc.). Multiple images were routinely obtained at focal planes just above and below the targeted process during the course of the time lapse image series experiments. Images that most closely matched in focus to the preceding images in the series were selected for quantification.

There is considerable variability in the criteria used to classify and distinguish dendritic spines and filopodia, some of which depend on ultrastructural analyses not available in the present study. Previous literature has classified dendritic protrusions that occur during early postnatal development into the following categories: filopodia, thin, stubby, and mushroom (Harris et al., 1992). Based on this classification, filopodia are long, thin protrusions that lack a head while thin protrusions are long like filopodia (>2 μ m) but have a head. Stubby spines lack a neck region but have a bulbous head. Mushroom spines are “mature” spines with a short length (<2 μ m), a clearly defined neck region, and a head that is >50% wider than the width of the neck (Lin et al., 2004). We will use the non-restrictive terms *process* or *protrusion* to encompass each of these types of dendritic structure. To measure the dimensions of dendritic processes, protrusions were divided into three regions: head, neck, and base (Fig. 4a). In cases where a clear head or neck region could not be defined, these parameters were not measured or included in the analysis. The head was measured as the widest region in the distal third of the protrusion. The neck width is measured as the widest region between the top and bottom thirds of the structure, between the neck and the base. The base width was measured at the widest region of the lower third of the structure near the shaft. The width of the head was determined by measuring the length of a line at the widest region of the spine head. This length was considered the diameter of the spine head and the radius was entered into the formula for calculating the volume of a sphere: $(4/3)\pi r^3$. Using

this calculation to assess the volume of the spine head assumes that spine head is a perfect sphere, and therefore results in an overestimate of the actual spine head volume, but serves a useful approximation for comparing processes. Total process length was calculated as the length of a straight line from the tip of the head of the protrusion to the point at which the protrusion merges with the shaft. The length of the neck was measured from the base of the head of the protrusion to the base of the protrusion. These dimensions were measured at the time points indicated in both stimulated and unstimulated protrusions. Unstimulated neighboring protrusions within 20 μm of the stimulated protrusion were measured to compare changes that occur without stimulation over the imaging period to the changes that occur due to photolysis of caged glutamate.

RESULTS

Development of postsynaptic protrusions in rats and mice *in vitro*

We first characterized the time course of dendritic process development and maturation qualitatively in organotypic hippocampal slice cultures prepared with the roller tube technique from images of GFP-filled protrusions acquired at various stages of postnatal development. In both rats and mice, we observed that a loss of immature protrusions and the appearance of mature spines took place over the second postnatal week of development. Pyramidal cells in rat cultures prepared from P5–7 rat pups were transfected with GFP biolistically at DIV 7–12 (= P12–19) to produce cytoplasmic filling. Pyramidal cells in rat cultures exhibited many long, thin immature protrusions at ages <P14 (Fig. 1, left) but they were largely absent by P19, when more mature looking spine-like processes predominated (Fig. 1A, right). Pyramidal cells in cultures prepared from GFP-expressing mice (GFP-m line, Feng et al., 2000) followed the same general developmental trend, however, even in cultures prepared from mice at P1, CA1 cell dendrites exhibited more mature-looking spines than immature protrusions by P10 than in rat cultures at equivalent ages (Fig. 1B, center), suggesting that these protrusions mature at a slightly earlier age in mice than in rats. In addition, filopodial structures that lacked a distinctive head were rare, even at the earliest stages of postnatal development (Fig. 1A, left).

Based on this developmental profile, we defined immature protrusions as long, thin structures >2 μm in length with either no head, or a head whose width was <50% greater than the width of the neck, so that the width of the head is similar to the width of the neck (Lin et al., 2004). This definition thus includes structures that have been defined in previous fluorescence time-lapse imaging studies as filopodia, protospines, and/or thin spines (Dailey and Smith, 1996; McKinney et al., 1999; De Roo et al., 2008). We defined mature protrusions, in contrast, as mushroom-shaped processes that were <2 μm in length, with a distinct head that was >50% wider than the width of the neck (Lin et al., 2004).

Immature protrusions lack AMPARs

Excitatory photolytic currents (phEPSCs) were evoked in hippocampal CA1 pyramidal neurons by focally photolyzing caged glutamate at the heads of dendritic protrusions under whole-cell voltage-clamp (−70 mV; Bagal et al., 2005), while simultaneously visualizing the protrusion using time-lapse recording of fluorescence images. Slice cultures were prepared

from P5–7 rats and maintained for 2–10 days *in vitro* and whole-cell recordings were performed with Alexa dye in the pipette solution in order to visualize the processes of the patched pyramidal cell. Because the photolysis-induced currents recorded (phEPSCs) are generally less than 20 pA (Bagal et al., 2005), protrusions <50 μm from the soma were stimulated in order to minimize signal decay due to dendritic filtering.

Inward currents with fast and slow components were elicited when a brief (1 ms) photostimulus was used to focally uncage glutamate at mushroom-shaped dendritic spines in immature tissue in Mg^{2+} -free ACSF. The fast component was observed in isolation after adding D-AP5 (40 μM) to block the slow component (Fig. 2A right), demonstrating that these phEPSCs were mediated by both NMDA and AMPA receptors. When glutamate was uncaged at immature protrusions in Mg^{2+} -free ACSF, in contrast, only slow, inward currents were recorded. These slow currents were abolished by application of D-AP5 (Fig. 2A, left), indicating that at least some immature protrusions lack AMPARs. We identified 5 immature protrusions lacking AMPARs in tissue from mice at P15 or earlier and did not identify immature protrusions lacking AMPARs in tissue older than P15 although the AMPAR-mediated current was smaller in immature protrusions than in mature protrusions. Consistent with previous reports (Matsuzaki et al., 2001; Zito et al., 2009), we found a strong correlation between the volume of the head of the stimulated protrusion and the amplitude of AMPAR-mediated phEPSCs recorded in the presence of AP5 (Fig. 2B left; P8–P10, $n=8$; P11–P14, $n=13$; $>P15=14$; Pearson's $r=0.777$, $p<0.05$). The AMPA:NMDA ratio was also strongly correlated with spine head volume (Pearson's $r=0.794$, $p<0.05$), but not directly with postnatal developmental age. That is, processes with the morphological characteristics of mature spines (large volume head, short length) exhibited large AMPAR phEPSC amplitudes and AMPA:NMDA ratios even at early postnatal stages. Furthermore, immature, filopodia-like processes possessed numerous NMDARs but appeared to have few, if any, AMPARs at their surface regardless of the age of the tissue.

Glutamate triggers morphological changes in immature protrusions

We hypothesized that stimulating the NMDA receptors of immature protrusions might initiate morphological changes that ultimately result in the acquisition of a mature spine morphology. To stimulate NMDA receptors in single spines using the photolysis of caged glutamate, we adapted the theta-burst stimulus (TBS) protocol that is widely used for the induction of long-term potentiation (LTP) because it mimics burst discharges in presynaptic CA3 cells (Larson et al., 1986; Rose and Dunwiddie, 1986). Briefly, five trains of 1.5-ms laser pulses (3 pulses at 100 Hz) were delivered to uncage glutamate near the tip of a stimulated immature protrusion in mouse slice cultures between P8–P15. This train was repeated five times with an inter-train interval of 200 ms. In ~39% of the stimulated processes (17/44), either minimal or no long-lasting morphological changes were detected after delivery of the TBS.

In the remaining experiments (61%, $n=27/44$), this stimulus protocol resulted in some or all of the following morphological changes to the immature process: a decrease in length (head and neck), an increase in the diameter of the head, an increase in the diameter of the neck, and an increase in the diameter of the base (Fig. 3B). In many cases, these changes were

accompanied by a branching of the process (Fig. 3C). In some experiments we also observed the emergence of small protrusions from the head, neck, base regions of the process itself, and/or the outgrowth of new protrusions from the dendritic shaft (Fig. 3A). In 8 of the 27 cases in which the immature protrusion demonstrated morphological changes, the process assumed a short, mushroom shape indistinguishable from a mature spine that persisted for at least 30 min (Fig. 3C–D).

The occurrence of at least one of the phenomena illustrated in Figure 3 was observed to varying degrees at each of the stimulated protrusions at which morphological changes occurred. To quantify these morphological changes, data obtained at the different observation times were normalized to the parameters measured immediately before the TBS was delivered for all of the responding processes ($n=27$; 4B–F). Significant changes in several measures of process length and width were observed consistently following the TBS protocol. The morphological changes were typically observed within 2–5 minutes of delivery of the TBS and persisted or continued to evolve for 30 min following stimulation. The significant increase in head width in immature processes that occurs after an uncaging stimulus in these experiments ($n=27$, $F_{1,108}=10.72$; $p<0.05$, one-way repeated measures ANOVA with Dunnett post-tests compared to pre-stimulation baselines, Fig. 4D) resembles the responses reported to accompany LTP in other studies (e.g., Matsuzaki et al., 2004). Protrusions that were within 100 μm of the stimulated protrusion did not demonstrate an increase in head width ($n=9$, Fig. 4D). In addition, both the total length and neck length of stimulated protrusions decreased after the TBS, consistent with the acquisition of mature spine morphology, while control protrusions did not exhibit a decrease in either parameter (Figure 4B–C).

Outgrowth of new processes was also a common result of the TBS. The outgrowth of processes occurred either from the stimulated protrusion itself as a branch or from the shaft confined to a region $<5 \mu\text{m}$ of the stimulated protrusion (Fig. 3A) within 2–5 minutes of stimulation. Outgrowth from the stimulated protrusion or the surrounding shaft occurred in 57% of the protrusions that exhibited morphological changes ($n=27$; Fig. 4G). The nature of the outgrowth varied between processes. In some experiments, the original process formed a new branch, whereas in others the outgrowth appeared to be a new process. New branches of the same process were formed from the shaft, near the base of the stimulated protrusion (2 out of 27, 7%) or from the neck region (5 out of 27, 19%; Fig. 3C). The most common form of outgrowth that occurred was the appearance of a spine head protrusion (SHP), a small protrusion extending from the head of the stimulated process (9 out of 27 or 33%). This result is consistent with previous reports of glutamate-induced outgrowth (Richards et al., 2005), although it has not been reported in studies employing glutamate photolysis in older tissue (e.g., Matsuzaki et al., 2004). In 6 out of 27 cases (22%), photolysis resulted in the outgrowth of new protrusions from the shaft, within 1 μm of the uncaging spot. These nascent protrusions lacked enlarged heads and thus resembled filopodia. In 4 of those cases, the nascent protrusion grew toward the protrusion at which the stimulus was delivered.

A novel form of morphological plasticity observed in these experiments was the increase in the width of the base and neck of the protrusions, which reached statistical significance after 2 and 10 minutes following the TBS, respectively ($F_{1,100}=2.99$, $p=0.02$; $F_{1,108}=2.87$,

$p=0.03$, one-way repeated measures ANOVA with Dunnett post-tests; Figure 4E,F). These changes reflect the appearance of a cytoplasmic ‘bulge’ in the process, first in the base and then in the neck. In experiments in which the process did not have a clear head prior to the delivery of the TBS, the bulge often moved to the distal end of the process, leading to the formation of a more pronounced head region (Figure 3C). Because endosomes supply the cargo necessary for LTP-induced structural plasticity of dendritic spines under some conditions (Cooney et al., 2002; Park et al., 2006), the cytoplasmic bulge that we have observed in response to TBS might be endosomes transporting PSD proteins to the head of the stimulated protrusion. Notably, this transport is not as pronounced in unstimulated neighboring protrusions ($n=9$, Figure 4E–F).

TBS-induced morphological changes and outgrowth are glutamate receptor-dependent

Previous studies have indicated the importance of NMDAR activation in the enlargement of head size in mature spines (Matsuzaki et al., 2004; Kopec et al., 2006; Harvey and Svoboda, 2007; Steiner et al., 2008; Yang et al., 2008) and the outgrowth of dendritic protrusions (Maletic-Savatic et al., 1999; Engert and Bonhoeffer, 1999). Because not every immature process responds morphologically to the TBS, a before and after design was used to test whether NMDARs were responsible for the types of morphological changes described in Figures 3 and 4. D-AP5 (80–160 μM) was bath applied and a first TBS was delivered. AP5 was then washed out from the bath 10 minutes later. Image acquisition was continued for 30 minutes to determine whether morphological changes occurred in the presence of AP5. A second TBS was then delivered in the absence of AP5, 30 min after the first TBS. This experimental design allowed us to determine which types of morphological changes were mediated by NMDAR activation (apparent after washout), and whether morphological changes occurred that were not due to NMDAR activation (apparent in AP5). Because not every process responds to the glutamatergic stimulation, only processes that responded before or after washout of AP5 were analyzed (46%; $n=28$ out of 61).

An example of one experiment is shown in Figure 5A. In this experiment, delivering the TBS protocol in AP5 resulted in the outgrowth of an SHP, whereas delivering TBS after the washout period resulted in an outgrowth of several long, thin processes from the shaft surrounding the stimulated protrusion. The results of this experiment indicate that SHPs are both non-NMDAR and NMDAR-dependent while the outgrowth of processes from the shaft is only NMDAR dependent.

Some morphological changes were observed in the presence of AP5, while others were not. To allow comparison between the morphological changes that occurred in AP5 and those that occurred after washout, data obtained at different observation times following TBS were normalized to the measured parameters at a time point preceding delivery of TBS in either condition (Fig. 5B–F). Consistent with results from previous studies (Matsuzaki et al., 2004; Kopec et al., 2006; Yang et al., 2008), AP5 prevented the significant increase in head size that occurred following TBS during the washout period ($n=21$, main effect of AP5: $F_{1,21}=27.42$, $p<0.01$, two-way repeated measures ANOVA with Tukey post-tests; Figure 5D). AP5 also prevented the decrease in whole length and the significant decrease in neck

length (main effect of AP5: $F_{1,21}$, $p < 0.05$) that occurred following TBS in the washout period (Figures 5B–C).

However, the mobilization of cytoplasmic bulges was observed to occur after TBS in AP5, though significantly less than after the washout of AP5 (Figure 5E and 5F). Similar to the results shown in figure 4, the base width increased significantly within two minutes following the delivery of TBS in the washout period (main effect of AP5: $F_{1,21} = 28.40$, $p < 0.01$; Fig. 5F) while the increase in neck width did not reach significance until five minutes following TBS in the absence of AP5 (main effect of AP5: $F_{1,21} = 4.34$, $p < 0.05$; Fig. 6E). Most notably, the transport in the majority of protrusions that exhibited morphological changes in AP5 occurred at the base (Figure 6F). This indicates that non-NMDARs, perhaps located at the shaft or base of the protrusion, may contribute to the structural plasticity of dendritic protrusions.

DISCUSSION

Stimulation of individual dendritic protrusions in developing organotypic hippocampal slice cultures with microphotolysis of caged glutamate was used to study the outgrowth and maturation of neuronal protrusions and dendritic spines and its dependence on glutamate receptor activation.

In the absence of external stimulation, we observed that immature protrusions (long $> 2 \mu\text{m}$, thin structures lacking a bulbous head) were replaced by mature spines ($< 2 \mu\text{m}$, head $> 50\%$ wider than the neck) in CA1 cells in organotypic hippocampal slice cultures from rats and mice over the second postnatal week. We also observed that cells in cultures prepared from Thy1-GFP mice exhibited mature spines at an earlier age than cells in cultures prepared from rats. The extent to which the preparation and culturing of the tissue as an explant influences these events is difficult to estimate, but our observations are consistent with the developmental time course described in intact tissue in previous studies (Harris et al., 1992; De Simoni et al., 2003).

Glutamate receptor content at immature protrusions

Conventional synaptic stimulation in the immature hippocampus triggers EPSCs that are mediated entirely by NMDARs, and not AMPARs, in some experiments (Isaac et al., 1995; Liao et al., 1995). The morphological correlate of such so-called silent synapses was not identified. We found that long, thin immature protrusions lacking clear heads responded to photolysis of caged glutamate with inward currents that contain no, or a very small, AP5-insensitive component, presumably because of a lack of AMPARs in their plasma membranes. This result is consistent with a recent study demonstrating that a subset of transient immature protrusions in the mature brain lack AMPARs (Zito et al., 2009). An alternative explanation for our results is that AMPARs are unstable at this stage, and can transiently appear at the surface of immature protrusions until they are stabilized by NMDAR activation (Xiao et al., 2004; Abrahamson et al., 2007).

We also found that the volume of the head of the stimulated protrusion was correlated directly with the amplitude of the AMPAR-mediated phEPSC and the AMPA:NMDA ratio

of the photolysis-induced response. In particular, we observed that the size of the AMPAR-mediated response depended only on the morphology of the protrusion and not on its developmental age. That is, processes that appeared morphologically mature displayed a more mature electrophysiological response with bigger AMPAR- and NMDAR-mediated components than morphologically immature processes in tissue of the same developmental age. We can thus conclude that the physiological and morphological maturation of spines is not driven by an intrinsic, cell autonomous developmental program, but rather the maturation process must occur in some manner that is unique to each individual protrusion.

Glutamate receptor activation promotes protrusion-specific structural changes

What process could drive the maturation of dendritic protrusions in a process specific manner? During early development, axons release glutamate from mobile clusters containing many components of presynaptic terminals (Kraszewski et al., 1995; Ahmari et al., 2000; Friedman et al., 2000). We hypothesized that the local release of glutamate from these release sites might provide a signal that promotes genesis of mature spines or maturation of immature processes. We tested this hypothesis by activating glutamate receptors at individual dendritic processes with microphotolysis of caged glutamate. We observed that bursts of photostimuli led to structural rearrangements of immature protrusions in many cases, so that they assumed a more mature morphological appearance, such as a shortening of the spine neck and the appearance or widening of the spine head. Our observations are thus consistent with the hypothesis that immature protrusions are the precursors of dendritic spines, both *in vivo* and *in vitro* (Morest, 1969; Dailey and Smith, 1996; Ziv and Smith, 1996; Maletic-Savatic et al., 1999; Marrs et al., 2001; Knott et al., 2006; De Roo et al., 2008) and that glutamate promotes this maturation. Not every stimulated process responded to photostimulation with a morphological change. Failure to respond may reflect inadequate stimulation or some intrinsic inability to respond. Our observations are consistent with the recent report suggesting that as the tissue matures it becomes more resistant to brief glutamatergic stimuli (Kwon and Sabatini, 2011).

We also observed the outgrowth of new immature, filopodia-like protrusions directly from the shaft in 6 of 27 experiments in which there was a morphological response and the formation of branches and spine head protrusions from the stimulated protrusion. Unlike Kwon and Sabatini (2011), we did not observe direct outgrowth of mature-looking, mushroom-shaped spines from the dendritic shaft in our experiments, perhaps because of differences in the intensity of the stimulation delivered.

While an increase in head size was commonly observed in our study, we also observed widening of the neck or the base of the protrusion in response to stimuli delivered to the spine head. Although the resolution of conventional light microscopy is much lower than that achieved using so-called super-resolution techniques (e.g. Nägerl and Bonhoeffer, 2010; Nägerl et al., 2007), we measured and then normalized the widths of the spine necks before and after photostimulation to provide an estimate of the changes that occur in the spine necks due to cytoplasmic transport that was observed upon delivery of TBS. In many cases, the width of the cytoplasmic bulges in the neck or base of the spine were comparable in width to the widths of the heads of spines, which are greater in width than the limits of our

resolution. Thus, our measurements are likely to underestimate the changes in neck width that occur after the TBS protocol.

Although we have shown previously that single stimuli activate receptors on the head of the targeted spine, the diffusion of glutamate away from the site of stimulation may be greater after trains of stimuli. It is thus possible that glutamate receptors may be present along the length of the protrusion and influence the response of the stimulated protrusion. Indeed, Fiala et al. (1998) reported that filopodial processes can form ultrastructurally detectable synapses along their length, and not just at their tips. We speculate that once glutamate receptors on the immature process have been activated and the proteins and receptors needed to form a mature synapse have been acquired, structural changes might then occur so that this cargo is obtained from the dendritic shaft and transported along the length of the immature protrusion. In some cases, this cargo is deposited at the head of the maturing dendritic spine, where, presumably, a mature PSD can then form.

We followed processes for only about 1 hr after stimulation, so we do not know what the fate of these processes might be. Newly formed processes are known to be unstable, and are prone to retraction at early stages of development (Holtmaat et al., 2005; De Roo et al., 2008; Zito et al., 2009). However, new processes make contact with presynaptic boutons rapidly in response to LTP-inducing synaptic stimuli (Nägerl et al., 2007), and may encounter the stabilizing influences of signaling proteins in the membranes of presynaptic boutons and astrocytes, such as ephrins and cell adhesion molecules, as well as signaling molecules in the extracellular matrix to which the nascent dendritic protrusion is exposed (Scheiffele et al., 2000; Chavis and Westbrook, 2001; Dansie & Ethell, 2011).

Glutamate receptor-induced morphological changes are partially NMDAR dependent

Developing axons release glutamate (Kraszewski et al., 1995; Ahmari et al., 2000) and NMDA receptor antagonists affect normal synaptogenesis *in vivo* (Rocha and Sur, 1995) and *in vitro* (Collin et al., 1997; Liao et al., 1999; Goldin et al., 2001). Our results, together with those of Kwon and Sabatini (2011), indicate a prominent role for NMDARs as mediators of glutamate-induced spine growth during development, which is also consistent with LTP-associated structural plasticity in more mature tissue (see Alvarez and Sabatini, 2007 for review). The NMDAR is ideally suited to serve as the glutamate sensor in immature processes because its high affinity for glutamate will allow it to respond to a gradient of glutamate released at some distance away and because of its ability to engage the postsynaptic biochemical signaling machinery. NMDAR activation is a primary trigger not only of physiological maturation in the form of AMPAR insertion (Isaac et al., 1995; Liao et al., 1995), but also anatomical maturation.

We did observe that some morphological responses, however, were not prevented by blocking NMDARs. The outgrowth of spine head protrusions, for example, appears to be NMDAR-independent, as also suggested by Richards et al. (2005). There is evidence that mGluR activation triggers growth of mature dendritic spines (Vanderklish and Edelman, 2001). It must also be noted that spines apparently can still form in the absence of synaptic transmission (Verhage et al., 2000), suggesting that there are parallel pathways driving spine outgrowth and maturation.

In conclusion, our observations support a model in which transient, motile filopodia respond to glutamate released from growing immature axons. These filopodia lack AMPARs, but respond to activation of their NMDARs with a variety of morphological changes that favor their transition to a more mature morphological and physiological state in which axo-spinous synapses express high levels of AMPA and NMDA receptors.

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Figure 1. Transition from filopodia to dendritic spines in CA1 pyramidal cells in hippocampal slice cultures. **A**, Images of apical dendritic segments in GFP-transfected CA1 cells in organotypic hippocampal slice cultures made from P7 rats. Most dendritic processes were thin, elongated structures with morphology typical of immature protrusions (open arrowheads) at P13 and P15, whereas at P19 the majority of the protrusions exhibited a mature spine morphology. (Scale bar, 1 μ m). **B**, Dendritic protrusions in organotypic hippocampal slice cultures made from thy1-GFP mice at P1 were mostly mature spines in

organotypic hippocampal slice cultures already at P7, the earliest age at which we could visualize them.

Figure 2.

A, phEPSCs recorded from a long, thin immature process (left), a “thin” spine (middle) and a “stubby” spine (right) during the second PN week in Mg^{2+} -free saline before and after application of AP5. Scale bar = $1\ \mu\text{m}$. B, Correlation of spine head volume with AMPAR-mediated responses to photostimulation (left) and the ratio of AMPAR- to NMDAR-mediated responses (right) in processes of various postnatal ages. Note that both physiological parameters are dependent on spine head size, not postnatal age. (P8–P10, $n=8$; P10–P14, $n=12$; >P15, $n=13$; Pearson’s $r=0.79$, $p<0.001$).

Figure 3.

Morphological responses of immature dendritic processes to glutamate photostimulation. **A**, Photostimulation with the TBS protocol ($t=0$) led to the outgrowth of processes from the shaft near the stimulated protrusion **B**, Photomicrographs of immature protrusions exhibiting cytoplasmic bulges at different points along the length that were mobilized (top) or enlarged (bottom) after delivery of TBS ($t=0$). **C**, Examples of processes that became more spine-like and branched after TBS. **D**, Example of a process that exhibited a large increase in head size in response to TBS. Scale bar = 1 μm .

Figure 4.

Quantification of glutamate-induced morphological responses. The length and width of the stimulated protrusion was measured 5 min before stimulation (–5), Immediately prior to stimulation (0), and at time points up to 30 min following stimulation for all processes that exhibited a morphological response to the TBS stimulation (indicated by dashed vertical line). The length or width of the process was then normalized to the average length or width at –5 min and 0. **A**, Cartoon illustrating the points along a dendritic process at which the various morphological parameters were measured. **B–F**, Following the delivery of the TBS

protocol, the total length of the protrusions ($F_{1,110}=4.19$, $p<0.01$) (**B**) and their neck length $F_{1,110}=3.25$, $p<0.01$ (**C**) decreased persistently, while their head width ($F_{1,108}=10.72$, $p<0.01$) (**D**), neck width ($F_{1,108}=2.87$, $p=0.03$) (**E**), and base width ($F_{1,100}=2.99$, $p=0.02$) (**F**) all increased. ($n=27$). * $p < 0.05$, one-way repeated measures ANOVA with Dunnett post-tests. **G**, Many new processes often extended from various locations of or near the stimulated process in response to the TBS stimulation. This outgrowth occurred in the form of spine head protrusions (SHPs) from the head, or processes that extended from the neck, or shaft near ($<50\ \mu\text{m}$) the stimulated protrusion.

Figure 5.

Some, but not all, glutamate-induced morphological responses are NMDAR-dependent. **A**, Experimental design is illustrated above photomicrographs of an immature protrusion stimulated with the TBS protocol in the presence of D-AP5 (80–160 μ M), and following washout. **B–F**, Length and width measures of stimulated protrusions in the presence of AP5 (black) and following washout (gray). Changes in head and neck length were inhibited by AP5, as was the increase in head width (main effect of AP5, $F_{1,21}=27.42$, $p<0.001$; $F_{1,21}$, $p<0.05$, respectively), whereas other changes were largely resistant to AP5 ($n=21$). * $p < 0.05$.

two-way repeated measures ANOVA with Tukey post-tests. **G**, Outgrowth at various locations was decreased in AP5 but increased following washout.