Neuron, Vol. 38, 773–784, June 5, 2003, Copyright ©2003 by Cell Press

Bidirectional Regulation of Hippocampal Mossy Fiber Filopodial Motility by Kainate Receptors: A Two-Step Model of Synaptogenesis

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Summary

The rapid motility of axonal filopodia and dendritic spines is prevalent throughout the developing CNS, although the function of this motility remains controversial. Using two-photon microscopy, we imaged hippocampal mossy fiber axons in slice cultures and discovered that filopodial extensions are highly motile. Axonal filopodial motility is actin based and is downregulated with development, although it remains in mature cultures. This motility is correlated with free extracellular space yet is inversely correlated with contact with postsynaptic targets, indicating a potential role in synaptogenesis. Filopodial motility is differentially regulated by kainate receptors: synaptic stimulation of kainate receptors enhances motility in younger slices, but it inhibits it in mature slices. We propose that neuronal activity controls filopodial motility in a developmentally regulated manner, in order to establish synaptic contacts in a two-step process. A two-step model of synaptogenesis can also explain the opposite effects of neuronal activity on the motility of dendritic protrusions.

Introduction

The formation of synaptic connections, an event central to the specificity of neural circuits and the subsequent computational function of the brain (Ramón y Cajal, 1923), is still poorly understood (Jessell and Sanes, 2000). Although neuronal activity can regulate the refinement of neural circuits (Katz and Shatz, 1996; Wiesel, 1982) and the growth and stabilization of axonal and dendritic structures (Cline, 2001), the formation of some early synaptic contacts per se does not appear to require synaptic activity (Verhage et al., 2000). At the same time, throughout the nervous system, during the period of synaptogenesis, pre- and postsynaptic structures show rapid motility over a time scale of seconds (Chang and De Camilli, 2001; Dunaevsky et al., 1999; Fischer et al., 1998; Lendvai et al., 2000; Wong et al., 2000). This rapid, actin-based motility is thought to be involved in synapse formation and rearrangement (Bonhoeffer and Yuste, 2002; Dunaevsky and Mason, 2003) and may even occur in more mature animals (Trachtenberg et al., 2002; but see Grutzendler et al., 2002).

The role of neuronal activity in the regulation of pre- and postsynapic motility is controversial. While some studies indicate that neuronal activity can *stimulate* motility and growth of dendritic protrusions (Engert and Bonhoeffer, 1999; Fiala et al., 2002; Maletic-Savatic et al., 1999; Toni et al., 1999; Wong et al., 2000), other experiments show that excitatory transmission may actually *inhibit* dendritic and axonal motility (Chang and De Camilli, 2001; Fischer et al., 2000). As a potential solution to this controversy, similar to the proposal for the dependency of growth cone motility and calcium (Kater et al., 1988; Gomez and Spitzer, 2000), the role of activity on the dynamics of dendrites and axons could be complex, with different levels of activity producing growth or retraction of dendritic (or axonal) protrusions (Harris, 1999; Kater et al., 1988; Matus, 2000; Segal and Andersen, 2000; Wong and Wong, 2000; Yuste and Bonhoeffer, 2001).

To better understand the pre- and postsynaptic interactions during development, we examined the role of neuronal activity in regulating the motility of axonal filopodia of hippocampal mossy fibers, the axons from dentate granule cells that extend into the CA3 region. Mossy fibers develop postnatally and produce three types of terminals: (1) mossy terminals, which synapse with thorny excrescences of CA3 pyramidal neurons, (2) small en passant terminals, and (3) filopodial extensions from mossy terminals, which specifically contact interneurons (Acsady et al., 1998). Although dendritic and axonal filopodia are developmentally transient structures, hippocampal mossy fiber filopodia remain in the adult (Acsady et al., 1998). Mossy fibers contain kainate (KA) receptors (Represa et al., 1987; Schmitz et al., 2001) and most KA receptor subunits are highly expressed in dentate granule cells (Schmitz et al., 2001).

Here we describe the rapid motility of axonal filopodia from hippocampal mossy fibers in brain slice cultures using two-photon microscopy. Axonal filopodia show a high degree of motility on a time scale of seconds, and this motility is downregulated as development proceeds. At the same time, filopodia that are in contact with their postsynaptic targets, CA3 interneurons, are highly stable. We examined the role of neuronal activity in regulating this motility. Long-term blockade of KA receptors prevents the developmental reduction of filopodial motility. In addition, filopodial motility is bidirectionally regulated by synaptic stimulation, an effect mediated by KA receptors. Based on these data, we propose a two-step regulation of synapse formation by glutamate, whereby the effect of neuronal activity on protrusion motility could be dual, with a stimulatory effect that would help the axonal filopodia find its synaptic target and an inhibitory effect once the synaptic contact has been established.

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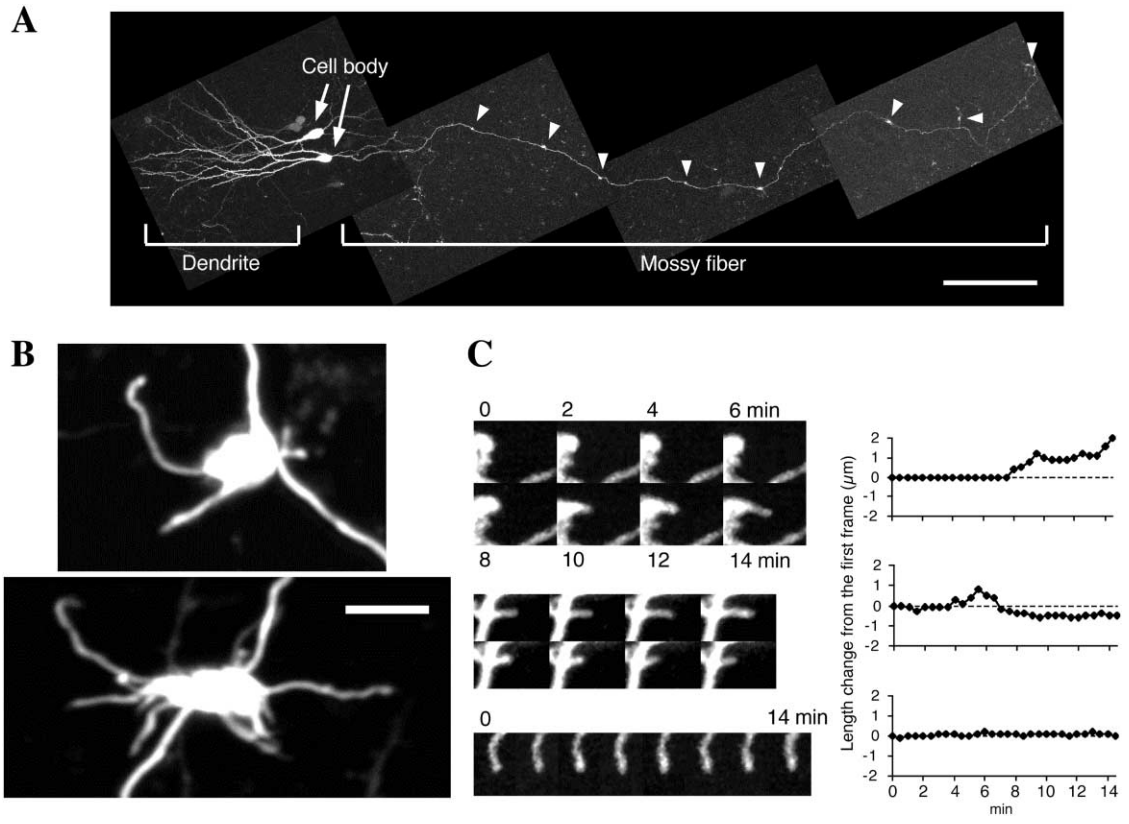

Figure 1. Motility of Mossy Fiber Filopodial Extensions

(A) Two-photon image of living dentate granule cells transfected with GFP. Note that their cell bodies, dendrites, and axons are clearly visible. Arrowheads indicate mossy terminals.

(B) Mossy terminals with filopodial extensions.

(C) Time-lapse imaging of filopodial extensions. Traces on the right show changes in filopodial length observed in sequences shown on the left. Plotted values are length at each time point subtracted from first time point.

Scale bar equals 150 μm in (A) and 5 μm in (B) and (C).

Results

Actin-Based Motility of Filopodial Extensions in Hippocampal Mossy Fibers

To visualize living mossy fiber axons from single granule cells, we transfected hippocampal slices prepared from neonatal mice and cultured for 2–3 weeks with EGFP, using biolistics (Tashiro et al., 2000). GFP expression was maximal 2–6 days after transfection, and dendrites and axons from granule cells (Figure 1A), including mossy terminals and filopodial extensions, were clearly labeled (Figure 1B). Up to 11 filopodia were associated with individual mossy terminals (13–15 days in vitro [DIV], 5.1 ± 0.3 filopodia; 20–22 DIV, 7.5 ± 0.7 filopodia), and the average length of a filopodium was 4.0 ± 0.1 μm at 13–15 DIV and 3.0 ± 0.3 μm at 20–22 DIV.

We examined the dynamics of mossy terminals and filopodia using a custom-made two-photon microscope (Majewska et al., 2000). We performed time-lapse imaging of these terminals over 15 min, with images taken every 30 s. We found that filopodial extensions, but not mossy or en passant terminals, were highly motile. Although some filopodia showed complex branching behaviors, similar to axonal growth cones (not shown), the predominant type of motility in axonal filopodia was elongation and/or retraction (Figure 1C). Therefore, we quantified this type of motility as changes in length of filopodia over time (see Experimental Procedures). Among filopodia, motility was highly variable (Figures 1C and 2A). The change in length was as rapid as 5.4 μm/min, although a significant number of filopodia did not move within the imaging period (15 min; Figures 1C and 2A). In addition, filopodial motility was actin based, since bath application of Cytochalasin D, an inhibitor of actin polymerization, blocked filopodial motility (1–2 μM; control, 0.105 ± 0.035 μm/min; CytoD, 0.030 ± 0.009 μm/min, $p < 0.05$, $n = 10$, paired t test).

To test whether filopodial motility was an artifact of long-term slice culturing, we also examined filopodia in acute hippocampal slices. Hippocampal slices were prepared from P14 mice and labeled with DiOlistics (Gan et al., 2000). DiO-labeled cells were imaged and their filopodia showed motility which was indistinguishable from that in long-term slice culture preparation at the same range of age (culture at 13–15 DIV, 0.036 ± 0.004 μm/min, $n = 185$; acute at P14, 0.041 ± 0.028 μm/min,

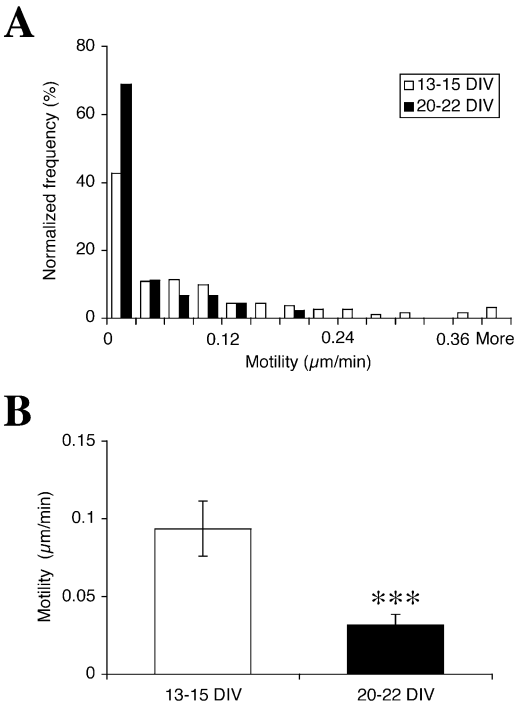

Figure 2. Filopodial Motility Is Developmentally Regulated

(A) Differential distribution of filopodial motility at 13–15 DIV and 20–22 DIV.

(B) Mean motility of filopodial extensions at 13–15 DIV and 20–22 DIV. Motility is significantly reduced during this period (13–15 DIV, 0.093 ± 0.009 μm/min, n = 185; 20–22 DIV, 0.031 ± 0.006 μm/min, n = 45; $p << 0.000001$, t test).

p = 0.88, n = 3, t test; 5 min interval imaging; see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/38/5/773/DC1).

Developmental Regulation of Filopodial Motility

To examine the developmental change of filopodial motility, we compared filopodial motility in slices kept for 2 versus 3 weeks in vitro. At 13–15 DIV, most filopodia were highly motile (0.093 ± 0.009 μm/min, n = 185 filopodia; Figure 2A). Approximately 40% of filopodia were stable, and motility of the remaining filopodia ranged up to 0.868 μm/min. In contrast, at 20–22 DIV, motility was significantly reduced (0.031 ± 0.006 μm/min, n = 45; $p < 0.001$, t test; Figures 2A and 2B), 70% of filopodia were stable, and no filopodia showed motility greater than 0.2 μm/min. Thus, the primary change between 2 and 3 weeks in vitro is the conversion of highly motile to nonmotile filopodia (Figure 2A).

Filopodial Motility Is Inversely Correlated with Contact with Target Cells

The developmental reduction in filopodial motility suggested that filopodia might stabilize due to synapse formation. To investigate this, we examined the relationship between filopodial motility and the existence of contacts with surrounding cells. Mossy fiber filopodia make synapses with CA3 interneurons (Acsady et al., 1998), about ~30% of which are parvalbumin or calretinin positive (Freund and Buzsaki, 1996). Because of this, imaged slices were fixed and immunostained with anti-parvalbumin and anti-calretinin antibodies and reconstructed with a confocal microscope. At 13–15 DIV, we found nine filopodia whose tips were closely associated with immuno-positive dendrites (Figures 3A and 3B). We then compared the motility of these filopodia with that of the remaining filopodia in the same movies. Filopodia contacting interneurons showed significantly less motility than filopodia without contact (with contact, 0.026 ± 0.020 μm/min, n = 9; without contact, 0.128 ± 0.017 μm/min, n = 29, $p < 0.002$, t test; Figure 3C). Although the resolution of confocal microscopy is not sufficient to confirm synaptic contacts, less motility in filopodia contacting interneurons suggests that filopodia making synaptic contacts are stabilized.

We also immunostained the imaged slices with an anti-PSD95 antibody, a postsynaptic marker (Marrs et al., 2000). Because of densely packed neuropil in slices, it was difficult to isolate clear immunostaining for PSD95. Nevertheless, we succeeded in detecting one filopodium unambiguously associated with a PSD95-immunopositive region (Figure 3D). This filopodium was completely stable (0 μm/min), further supporting the idea that filopodia with synaptic contacts are stable.

Filopodial Motility Is Correlated with Free Extracellular Space

To further understand the relationship between filopodial motility and surrounding cellular processes, we performed ultrastructural reconstructions of the same filopodia imaged by two-photon time-lapse movies (Figure 4; Dunaevsky et al., 2001). We reconstructed eight imaged filopodia as well as five nonimaged filopodia from the same slices (Figures 4A–4D). The reconstructed filopodia did not have mature synapses, as characterized by a thick postsynaptic density and clustered presynaptic vesicles. However, the tips of most filopodia contained synaptic vesicles that were not clustered. Also, in cells contacted by some filopodia, membrane specializations with electron-dense material were apposed to filopodial tips (Figures 4B and 4C). These specializations have been considered morphological signatures of immature synapses (Vaughn, 1989). In addition, we found that filopodial motility was greater when there was more free extracellular space surrounding their tips ($p < 0.04$, linear regression; Figure 4E). These data support the hypothesis that motile filopodia are stabilized and stop moving when they contact their synaptic targets.

Developmental Reduction of Filopodial Motility Is Dependent on Kainate Receptors

We next examined the role of neuronal activity in the developmental regulation of filopodial motility. Since mossy fibers release glutamate (Schmitz et al., 2000, 2001), we performed long-term blockade of glutamate receptors with their antagonists during development. Slices were incubated with either an AMPA/KA receptor antagonist, CNQX (10 μM), or an AMPA-specific antagonist, GYKI53655 (12.5 μM), for 3 days and imaged at 16 DIV (Figure 5A). In control condition, filopodial motility was significantly reduced in this time period (Figure 5B;

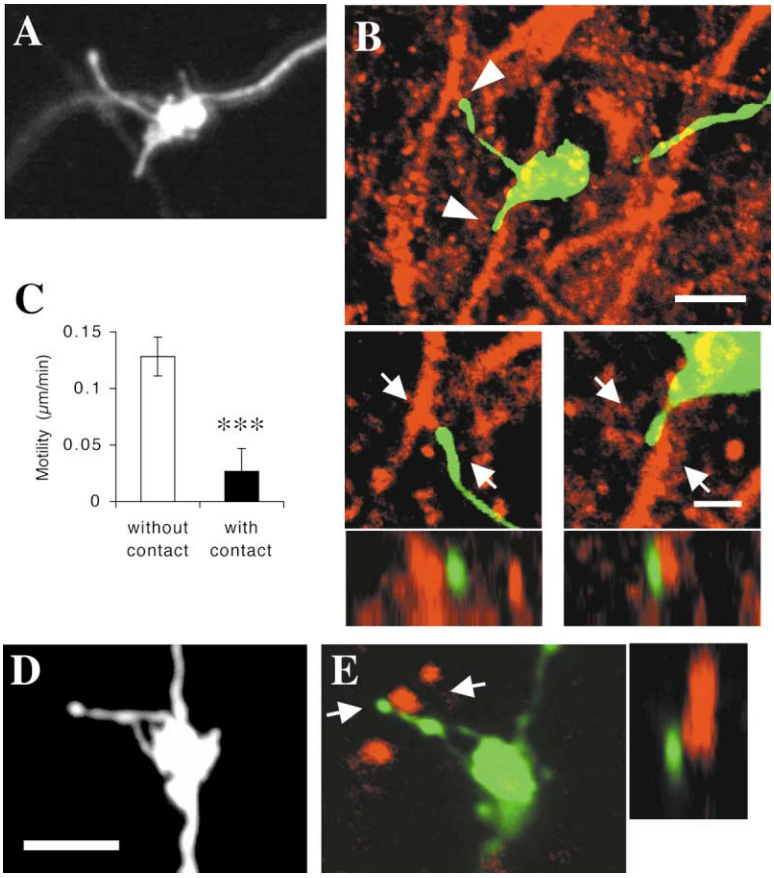

Figure 3. Filopodia Contacting Postsynaptic Targets Are Stable

(A and D) Two-photon images of mossy fiber axon terminals in a live slice culture (14 DIV).

(B) Confocal images of the same terminals as in (A), projected from three confocal sections. Two filopodia are contacting interneuron dendrites (top, arrowheads). The previously imaged slices are immunostained with anti-parvalbumin antibody. Green, GFP in mossy fiber; red, Cy3 in parvalbumin positive interneurons. Middle panels show high-magnification images of these two filopodia in single sections. Low panels show orthogonal sections whose positions are marked with arrows in middle panels.

(C) Average motility of nine filopodia contacting interneurons and filopodia without contact. Filopodia contacting interneurons show significantly less motility (with contact, 0.026 ± 0.020 μm/min, $n = 9$; without, 0.128 ± 0.017 μm/min, $n = 185$, $p < 0.002$, t test).

(E) Left: a confocal single-section image of the same terminals as in (D). Right: an orthogonal section whose positions are marked with arrows in left panels. Imaged slices are immunostained with anti-PSD-95 antibody. A filopodium closely associated with PSD-95 puncta was completely stable.

13 DIV, 0.116 ± 0.017 μm/min, $n = 24$; 16 DIV, 0.061 ± 0.012 μm/min, $n = 41$; $p < 0.02$, t test). This developmental reduction was blocked by CNQX, but not by GYKI53655 (Figure 5B; CNQX, 0.106 ± 0.017 μm/min, $n = 46$, $p = 0.68$; GYKI53655, 0.062 ± 0.013 μm/min, $n = 30$, $p < 0.03$, t test against control at 13 DIV), indicating that developmental reduction of filopodial motility and synapse formation is dependent on KA receptor activation.

Bidirectional Regulation of Filopodial Motility by Kainate Receptor Activation

To study how KA receptor activation mediates the developmental reduction of filopodial motility, we then examined short-term effects of KA receptor activation on filopodial motility at 13–15 DIV. In these experiments, we first imaged filopodial motility for 15 min in control condition and then carried out a second 15 min imaging session 20 min after drug application. First, we tested the effects of glutamate on motility and found that 100 μM glutamate significantly increased motility (0.167 ± 0.054 μm/min change, $p < 0.01$, $n = 22$, paired t test; Figure 6A). We then investigated the effect of specific glutamate receptor agonists (0.3–10 μM KA, 2 μM AMPA, 2 μM NMDA, and 100 μM ACPD) and found that only KA, an AMPA/KA receptor agonist, had significant effects on filopodial motility. Interestingly, the effect of KA was dose dependent (Figure 6A). Whereas 10 μM KA blocked filopodial motility (-0.097 ± 0.023 μm/min change, $p < 0.003$, $n = 11$), 1 μM KA induced a 2-fold increase in motility (0.097 ± 0.035 μm/min change, $p < 0.02$, $n = 18$, paired t test). Application of 3 μM KA promoted motility, but not significantly ($p = 0.12$, $n = 16$), and 0.3 μM KA had no effect on filopodial motility ($p = 0.90$, $n = 27$). Whereas motility induction by 1 μM KA occurs within 1 min after KA application, 10 μM produces a fast increase, followed by a decrease in motility after >5 min. This could be due to the initially lower concentration of KA reached during the perfusion exchange, and then higher concentration inhibits motility (Figure 6B). We excluded the possibility that the 10 μM KA blockade of filopodial motility was due to cell damage because motility was restored after a 1 hr washout (control, 0.104 ± 0.025 μm/min; washout, 0.087 ± 0.037 μm/min; $p = 0.69$, $n = 10$, paired t test; Figure 6C). We examined the specificity of the effects of KA using CNQX (50 μM), which blocked both the motility induction by 1 μM KA (-0.027 ± 0.039 μm/min change, $p < 0.03$, $n = 19$, t test; Figure 6D) and its inhibition by 10 μM KA (-0.015 ± 0.031 μm/min change, $p < 0.05$, $n = 19$, t test; Figure 6E).

We then examined the effects of KA in the presence of GYKI53655 (25 μM), which did not block either motility induction (0.088 ± 0.041 μm/min change, $p = 0.86$, $n = 19$, t test; Figure 6D) or its inhibition (-0.077 ± 0.036 μm/min change, $p = 0.63$, $n = 37$, t test; Figure 6E). Therefore, activation of KA receptors, but not AMPA receptors, mediates the bidirectional (i.e., stimulation or inhibition) effects of KA on filopodia motility.

We questioned whether these bidirectional effects of KA receptor activation were restricted to a particular

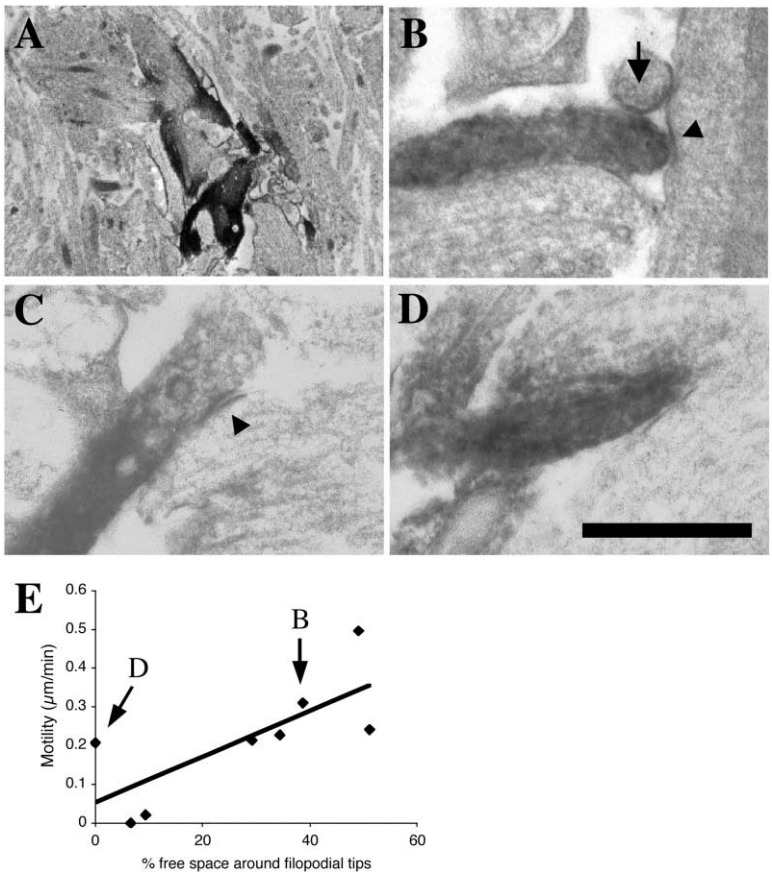

Figure 4. Filopodial Motility Is Correlated with the Amount of Free Extracellular Space

(A) The mossy fiber axons were immunostained by anti-GFP antibody. Dark staining represents an immunostained mossy fiber terminal.

(B and C) Filopodia with a putative immature synapse. These filopodia are apposed to electron-dense specializations on dendrites (arrowheads) and contain synaptic vesicles. Note neighboring unstained filopodium with vesicles (arrow in A).

(D) A filopodium completely surrounded by neuropil.

(E) Relation between filopodial motility and free extracellular space. For filopodia imaged in time-lapse movies and completely reconstructed in EM, motility is plotted against the percent free space around filopodial tips. The points corresponding to filopodia in (B) and (D) are marked. Scale bar equals 3 μm in (A) and 500 nm and (B)–(D).

developmental stage (13–15 DIV) or remained in more mature stages. Thus, we applied 1 or 10 μM KA to slices at 20–22 DIV and examined the effects on filopodial motility. We found that at older ages, 1 μM KA still increased filopodial motility (0.051 ± 0.023 μm/min change, $p < 0.006$, $n = 9$, t test; Figure 6A) and that 10 μM KA still inhibited motility (−0.072 ± 0.018 μm/min change, $p < 0.05$, $n = 14$, t test; Figure 6A). Since most filopodia at 20–22 DIV are stable (Figure 2A) and it is difficult to detect decrease in motility by analyzing the total population of filopodia, we only analyzed the effect of 10 μM KA on filopodia that already had significant motility (0.025–0.165 μm/min) in control condition. Thus, the bidirectional effects of KA receptor activation remained up to 3 weeks in vitro.

Mechanisms of the Effects of Kainate Receptor Activation on Filopodia Motility

We then focused on the mechanisms by which KA receptors regulate filopodial motility. To explore the role of Na^+-based action potential or voltage-sensitive Ca^{2+} channels (VSCC) in the regulation of motility by KA receptors, we examined the effects of KA in the presence of 1 μM TTX or 1 mM Ni^{2+}, respectively. For these experiments, we first incubated slices with antagonists for 20 min and then imaged filopodial motility to establish the control motility. We then added KA to the bath, and 20 min after incubation with KA, we performed another 15 min imaging session. We found different results using 1 μM or 10 μM KA (Figures 6C and 6D). Motility induction by 1 μM KA was blocked by Ni^{2+} (0.009 ± 0.017 μm/min change, $p < 0.02$, $n = 20$, t test) but not by TTX (0.047 ± 0.020 μm/min change, $p = 0.23$, $n = 39$, t test), whereas motility inhibition by 10 μM KA was blocked by TTX (−0.022 ± 0.026 μm/min change, $p < 0.05$, $n = 28$, t test against control) but not by Ni^{2+} (−0.188 ± 0.058 μm/min change, $p = 0.16$, $n = 17$, t test against control).

KA receptor activation has been hypothesized to modulate transmission of a subset of synapses through a Pertussis toxin-sensitive G protein in hippocampal CA1 region (Frerking et al., 2001; Rodriguez-Moreno and Lerma, 1998). We therefore examined the effect of Pertussis toxin on the modulation of filopodial motility by KA by preincubating slices with 5 μg/μl Pertussis toxin for 3 hr. Motility induction by 1 μM KA was insensitive to Pertussis toxin (0.080 ± 0.035 μm/min change, $p = 0.72$, $n = 23$, t test; Figure 6D), whereas motility inhibition by 10 μM KA was blocked by the toxin (0.031 ± 0.024 μm/min change, $p < 0.002$, $n = 13$, t test; Figure 6E). These results imply that different downstream mechanisms, potentially mediated by different types of KA receptors with different affinity, underlie the motility induction by 1 μM and inhibition by 10 μM, as well as rule out that motility inhibition is due to receptor desensitization.

Since KA receptor activation in hippocampal interneurons is known to depress GABAergic transmission (Rodriguez-Moreno and Lerma, 1998), it is possible that KA regulates filopodial motility indirectly by modulating

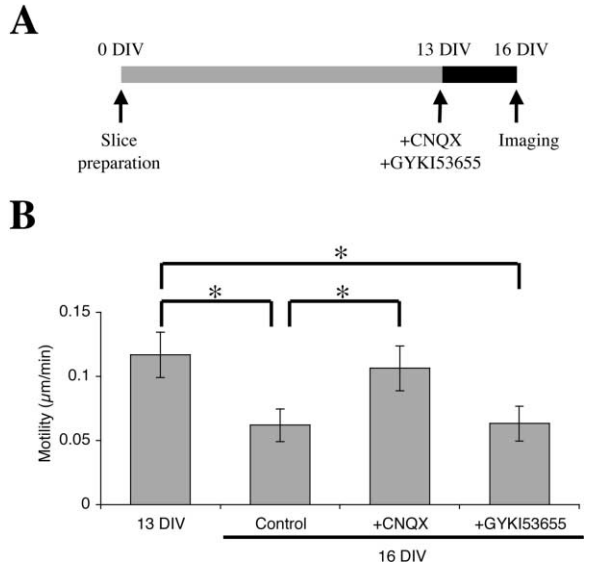

Figure 5. Developmental Reduction of Filopodial Motility Is Dependent on Kainate Receptor Activation

(A) Time course of the experiment. Slices are incubated with antagonists between 13 and 16 days.

(B) The average motility with 3 day blockade of glutamate receptors. In control condition, motility is significantly reduced between 13 and 16 DIV (13 DIV, 0.116 ± 0.017 μm/min, n = 24; 16 DIV, 0.061 ± 0.012 μm/min, n = 41, $p < 0.02$, t test). This developmental reduction was blocked by CNQX (0.106 ± 0.017 μm/min, n = 46, p = 0.68, t test against control at 13 DIV), but not by GYKI53655 (0.062 ± 0.013 μm/min, n = 30, $p < 0.03$, t test against control at 13 DIV). Difference in motility between control and CNQX is also significant ($p < 0.05$, t test). *$p < 0.05$ in t test.

transmitter release from interneurons. This might be the case for motility induction by 1 μM KA since the effect is inhibited by blocking synaptic transmission with 1 μM Ni^{2+}. Therefore, we examined the effect of 1 μM KA in the absence of GABAergic transmission, using 50 μM Picrotoxin, a $GABA_A$ receptor antagonist, and 20 μM SCH 50511, a $GABA_B$ receptor antagonist. To prevent epileptiform activity caused by blocking GABAergic transmission, APV and GYKI53655 were also applied. In these experiments, motility induction by 1 μM KA was not blocked (0.051 ± 0.036 μm/min change, p = 0.36, n = 26, t test against control; Figure 6D). These data suggest that transmitter release from interneurons is not involved in both motility induction and inhibition.

Schmitz et al. (2000, 2001) found differential effects of KA receptor activation on presynaptic excitability and transmitter release in mossy fiber. These bidirectional effects were mimicked by raising the extracellular K^+ concentration, suggesting that the depolarization of mossy fiber is involved in these effects. Therefore, we examined the effects of incubations with high concentration of extracellular K^+ on filopodial motility. We found that 6 mM K^+ increased motility, similarly to low concentration of KA (0.073 ± 0.025 μm/min change, $p < 0.02$, n = 14, t test; Figure 6F). However, higher concentration of extracellular K+ did not inhibit filopodial motility (12 mM, 0.010 ± 0.023 μm/min change, p = 0.66, n = 27, t test; 24 mM, −0.008 ± 0.017 μm/min change, p = 0.61, n = 20, t test; Figure 6F).

Regulation of Filopodial Motility by Synaptically Released Glutamate

Finally, we examined whether synaptically released glutamate has similar effects on filopodial motility using extracellular electrical stimulation with an electrode placed near the imaged mossy fiber terminals (<50 μm). We stimulated the terminals with 25 Hz continuously during the 15 min imaging period. At 13–15 DIV, filopodial motility was increased during electrical stimulation, compared to control conditions (control, 0.061 ± 0.009 μm/min; stimulation, 0.102 ± 0.012 μm/min, n = 39, $p < 0.05$, paired t test; Figure 7A, left). Thirty minutes after stimulation, filopodial motility was restored to control levels (0.063 ± 0.004 μm/min, p = 0.90, n = 39, paired t test; Figure 7A, left). This motility induction by electrical stimulation was also mediated by KA receptor activation since CNQX (50 μM) blocked it (−0.007 ± 0.011 μm/min change, $p < 0.04$, n = 22, t test; Figure 7A, right) but GYKI53655 (50 μM) and APV (100 μM) did not (0.046 ± 0.026 μm/min change, p = 0.87, n = 25; Figure 7A, right). To mimic the inhibition of motility produced by high concentration of KA, we performed similar experiments stimulating mossy fibers at higher frequencies, but we did not observe any inhibition of motility in slices of this age (13–15 DIV; data not shown).

We then asked whether this inhibition was present at 20–22 DIV. Since the majority of filopodia at these ages are very stable (Figure 2A), it is difficult to detect reduction in motility by analyzing the total population of filopodia. Therefore, we examined the effects of electrical stimulation (25 Hz) on filopodia that showed substantial motility in the time-lapse movies previously taken in control condition (0.030–0125 μm/min). We found that motility in these filopodia was significantly reduced during electrical stimulation (control, 0.089 ± 0.010 μm/min; stimulation, 0.012 ± 0.006 μm/min, n = 7, $p < 0.02$, paired t test; Figure 7B, left). The motility inhibition by electrical stimulation (control, −0.075 ± 0.025 μm/min change, n = 9) was also mediated by KA receptor activation since CNQX (50 μM) blocked this reduction (−0.005 ± 0.014 μm/min change, n = 6, $p < 0.04$, t test; Figure 7B, right) but GYKI53655 (50 μM) and APV (100 μM) did not (−0.082 ± 0.031 μm/min change, n = 4, p = 0.87; Figure 7B, right).

Discussion

Rapid Motility of Filopodia from Hippocampal Mossy Fibers

The motility of dendritic spines and axonal filopodia has been recently discovered by advances in imaging (Dailey and Smith, 1996; Ziv and Smith, 1996; Chang and De Camilli, 2001; Dunaevsky et al., 1999; Fischer et al., 1998; Lendvai et al., 2000; Wong et al., 2000) and remains, to a large degree, an exciting phenomenology in search of a function. Speculations on the potential role of spine and filopodial motility have ranged from epiphenomena of the actin cytoskeleton to regulation of critical periods or facilitating synaptogenesis (Bonhoeffer and Yuste, 2002; Dunaevsky and Mason, 2003). The regulation of this motility by neuronal activity is controversial, since experiments manipulating synaptic activity with glutamate or electrical stimulation have pro-

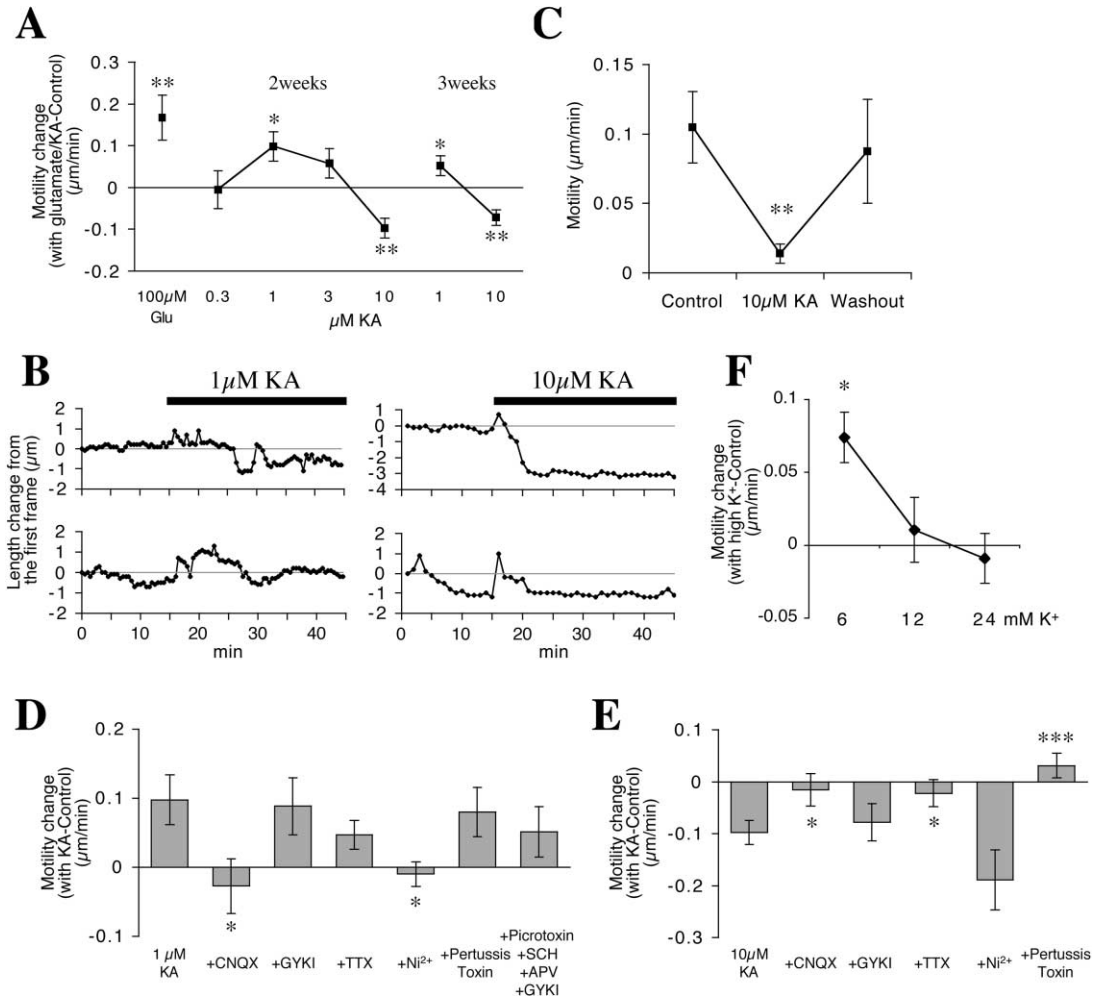

Figure 6. Bidirectional Regulation of Filopodial Motility by Kainate Receptors

(A) Effects of glutamate and kainate on filopodial motility. The effects of KA are dose dependent. Mean motility changes (motility with glutamate/KA − motility in control) are plotted for glutamate and different concentrations of KA at 2 and 3 weeks in vitro. Motility changes are significantly different from 0 with 100 μM glutamate and 1 μM and 10 μM KA at 2 and 3 weeks in vitro.

(B) Time course of the effects of KA on filopodia motility. Two representative examples of length change of filopodia are shown for 1 and 10 μM KA.

(C) Motility is restored after washout of KA. Motility in control condition, with 10 μM KA, and 1 hr after washout is plotted. Motility is reduced with 10 μM KA ($p < 0.006$, $n = 10$, paired t test). After 1 hr washout, motility is not significantly different from control ($p = 0.69$, paired t test).

(D and E) Mean motility change (motility with KA − without KA) in the presence of drugs designated below each column. (D) 1 μM KA; (E) 10 μM KA.

(F) The effects of high concentration of K^+ on filopodial motility. 6 mM K^+ significantly increases motility (0.073 ± 0.025 μm/min change, $p < 0.02$, $n = 14$, t test), whereas higher concentration (up to 24 mM) does not. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ in t test.

duced opposing results (Chang and De Camilli, 2001; Engert and Bonhoeffer, 1999; Fiala et al., 2002; Fischer et al., 2000; Maletic-Savatic et al., 1999; Toni et al., 1999; Wong et al., 2000). Similarly to the bidirectional dependency of growth cone motility on calcium concentration (Kater et al., 1988; Gomez and Spitzer, 2000), a potential solution to this controversy could be a dose-dependent or developmental stage-dependent effect of glutamate or synaptic activity on axonal filopodia or dendritic spine motility (Harris, 1999; Kater et al., 1988; Matus, 2000; Segal and Andersen, 2000; Wong and Wong, 2000; Yuste and Bonhoeffer, 2001).

Here we describe, for the first time, actin-based rapid motility of hippocampal mossy fiber filopodia. We show that there are two types of filopodia: one is highly motile, while the other is essentially stable. We find that filopodia contacting their postsynaptic targets, CA3 interneurons (Acsady et al., 1998), are significantly less motile than those that do not (Figure 3) and that motile filopodia are instead surrounded by free extracellular space (Figure 4). Based on these findings, we propose that the motility of filopodia mediates the search for their postsynaptic targets and that upon finding the correct targets, filopodia are stabilized. Thus, the function of filopodial motility may be to enable the exploration of the environment to find adequate postsynaptic targets (Ziv and Smith, 1996). The developmental reduction in filopodial motility due to the conversion of highly motile

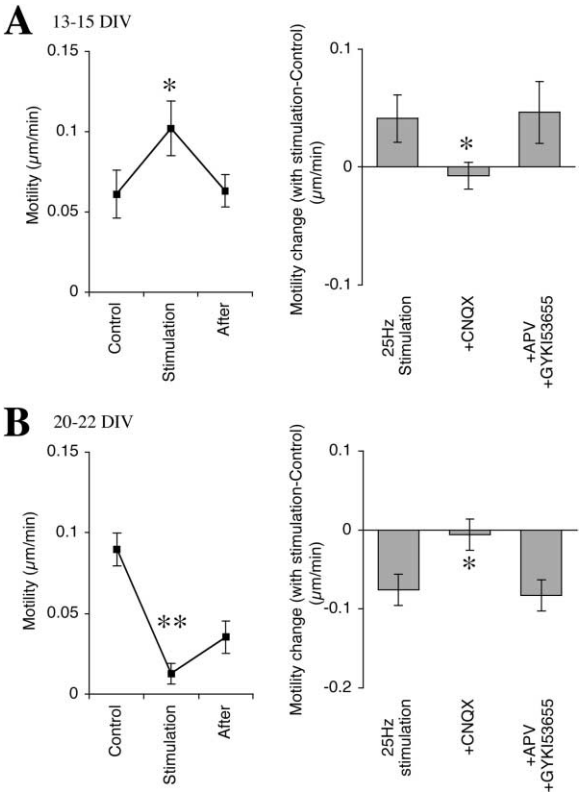

Figure 7. Synaptically Released Glutamate Bidirectionally Regulates Filopodial Motility through Kainate Receptors

(A) Left: at 2 weeks in vitro, electrical stimulation near imaged area (25 Hz, 10 V, within 50 μm) reversibly enhances filopodial motility. Motility is significantly increased during electrical stimulation (control, 0.061 ± 0.009 μm/min; during stimulation, 0.102 ± 0.012 μm/min, $p < 0.05$, paired t test). Right: electrical stimulation enhances filopodial motility through KA receptor activation. Mean motility changes (motility with stimulation − control) in the presence of drugs designated below each column. *$p < 0.05$ in t test.

(B) Left: at 3 weeks in vitro, the electrical stimulation protocol reversibly reduces motility in filopodia that show substantial motility in control condition. Motility is significantly decreased during electrical stimulation (control, 0.089 ± 0.010 μm/min; during stimulation, 0.012 ± 0.006 μm/min, $n = 7$, $p < 0.02$, paired t test). Right: electrical stimulation enhances filopodial motility through KA receptor activation. Mean motility changes (motility with stimulation − control) in the presence of drugs designated below each column. *$p < 0.05$ in t test.

filopodia into stable ones is likely, therefore, to reflect synapse formation.

Bidirectional Regulation of Filopodial Motility by Kainate Receptor Activation

We show that filopodial motility is bidirectionally regulated by KA receptor activation depending on the concentration of the agonist. Whereas lower KA concentrations induce filopodial motility, higher concentrations inhibit it. The regulation of filopodial motility by KA receptor activation is likely to be physiological since synaptically released glutamate induced by electrical stimulation also causes bidirectional effects on filopodial motility at different developmental stages. An inhibition of motility of axonal filopodia by glutamate was described in dissociated hippocampal neurons (Chang and De Camilli, 2001), suggesting it might be a widespread mechanism.

Since both induction and inhibition of filopodial motility are mediated by KA receptors, it is possible that the differential effects are due to receptor desensitization in the presence of high KA concentration. However, this is unlikely because the downstream mechanisms of these effects of KA seem to be quite different, suggested by differential sensitivity to three inhibitors, TTX, Ni^{2+}, and Pertussis toxin. For motility induction, KA receptor activation might cause a depolarization, which in turn could open VSCCs. The resulting Ca^{2+} influx could induce filopodial motility. In contrast, motility inhibition appears independent of VSCCs and may require additional depolarization by opening Na^{+} channels. Also, a metabotropic mechanism mediated by Pertussis toxin-sensitive subtypes of G protein (Rodriguez-Moreno and Lerma, 1998) appears involved in motility inhibition. Based on the differential effects by different concentration of KA and different downstream mechanisms, we hypothesize that the two effects of KA are mediated by different combinations of KA receptor subunits with different affinity. In fact, four KA subunits, GluR6, 7 and KA1, 2, are highly expressed in dentate granule cells (Schmitz et al., 2001) and form receptors with different affinities (Lerma et al., 2001). Further work is required to identify which subunits are involved in the bidirectional effects on filopodial motility.

Where are the KA receptors? Since mossy fibers contain presynaptic KA receptors (Represa et al., 1987), we favor the hypothesis that KA receptors on the filopodia, or other regions of mossy fiber axon, mediate the bidirectional effects. At the same time, CA3 interneurons also have KA receptors and we cannot rule out their involvement in regulating presynaptic motility. However, since the bidirectional effects are intact after blockade of transmitter release or GABA receptors, the involvement of GABAergic transmission is unlikely. Nevertheless, it is still possible that a factor other than GABA are nonsynaptically released from interneurons or other cell types in response to KA receptor activation and this factor could regulate filopodial motility.

Two-Step Regulation of Synapse Formation by Glutamate: A Model

The present data suggest that filopodia contacting postsynaptic targets show significantly less motility, implicating that motility of axonal filopodia underlies searching for postsynaptic targets. Then, once filopodia find correct targets, they appear to become stabilized, maintaining the contacts. This process appears to be regulated by KA receptor activation since the developmental reduction of filopodial motility, probably representing developmental increase of synapses, is inhibited by long-term blockade of KA receptors. In addition, KA receptor activation by synaptically released glutamate also regulates filopodial motility bidirectionally.

These data together suggest a two-step model of formation of synaptic contacts, whereby glutamate, released by the filopodia, regulates motility depending on the stages at which they are in the process of synapse formation (Figure 8). In immature filopodia, released glu-

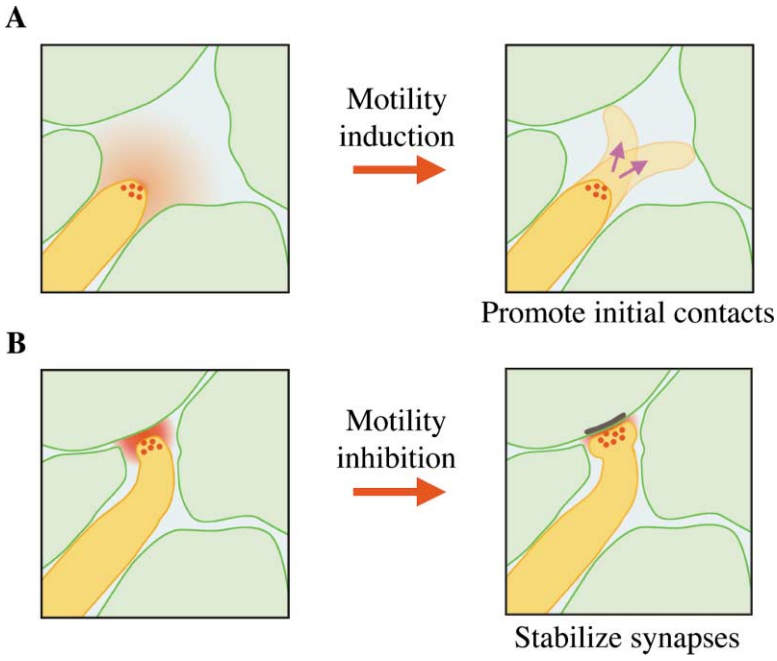

Figure 8. A Two-Step Model of Synaptogenesis of Axonal Filopodia

(A) In the first step, immature filopodia release glutamate, which could stimulate high-affinity KA receptors and, in turn, induces filopodial motility. In these immature filopodia without contacts, the induction of motility facilitates filopodia to explore their environment and contact potential synaptic targets.

(B) In the second step, more mature filopodia, which have a synaptic contact, release glutamate and activate low-affinity KA receptors such that filopodia are stabilized. The stabilized filopodia are facilitated to form mature synapses.

tamate could stimulate high-affinity KA receptors and, in turn, induce filopodial motility. In filopodia without contacts, the induction of motility could help them explore their environment and contact potential synaptic targets (Figure 8A). On the other hand, for filopodia in a more mature state, with a synaptic contact, released glutamate would activate low-affinity KA receptors or cause a sustained stimulation of the same high-affinity receptors, such that filopodia are stabilized and actually are prevented from moving. Our data showing that in older ages lower concentrations of kainate still enhance motility indicate that this bidirectional effect is likely due to a difference in the concentration of glutamate reached, but not change in the sensitivity of filopodia to glutamate. This stabilization of filopodia may facilitate the action of signaling molecules involved in synapse formation such as neuroligin (Scheiffele et al., 2000) or SynCAM (Biederer et al., 2002), promoting the formation of mature synaptic contacts (Figure 8B). Thus, although glutamate would regulate filopodial motility in both directions depending on the extent of KA receptor activation, the bidirectional regulation of motility could therefore facilitate synapse formation in two different steps. Our model assumes that basal level of motility is spontaneous and random and that synapse formation per se does not require synaptic activity (Verhage et al., 2000). However, synapse formation would be selectively facilitated on axonal filopodia with high degree of activity. Thus, overall the formation of functional neuronal circuits would be facilitated by neuronal activity.

A Potential Role for Extracellular Space in Controlling Motility and Synaptogenesis?

How could glutamate have differential effects on motility? How could different types or numbers of KA receptors become activated at different stages of synapse formation? As discussed above, the bidirectional effect of KA receptor activation is likely due to a difference in the concentration of glutamate reached, but not change in the sensitivity of filopodia to glutamate. This could be explained by differences in intrinsic properties of filopodia at different developmental stages, such as differential amounts of glutamate release or of release probability. Another possibility could be a difference in the physical environment filopodia encounter, including differences in the amount of free extracellular space, which would influence glutamate diffusion, or difference in glutamate clearance by glutamate transporters. The former possibility is consistent with our ultrastructural finding that filopodial motility is correlated with free extracellular space around filopodia. Free space would allow rapid dilution of glutamate so that glutamate concentration around filopodia would decrease quickly and activate only high-affinity receptors. In contrast, limited space around mature filopodia may maintain higher concentrations of glutamate and thus activate low-affinity receptors. Thus, axonal filopodia could use their own released glutamate as a sensor to probe their immediate environment and facilitate encountering synaptic targets and stabilize synapse formation. Further experiments are necessary to examine these possible mechanisms of differential activation of different KA receptors.

Why would mossy fiber filopodia be so motile? Since these filopodia specifically contact interneurons (Acsady et al., 1998), which are presumably harder to encounter in the neuropil compared with dendrites of CA3 pyramidal neurons, it seems reasonable that their motility could help them identify their specific target. Therefore, filopodial motility could implement synapse specificity.

Filopodial Motility in the Adult Brain

Morphological rearrangements of dendritic spines in the adult cortex has been recently described by Trachtenberg et al. (2002), although these data are controversial (Grutzendler et al., 2002) and it is unclear yet whether

there is significant morphological plasticity in the adult CNS. We find interesting that, whereas axonal and dendritic filopodia in other types of neurons are restricted during the period of synaptogenesis (Bonhoeffer and Yuste, 2002), mossy fiber filopodia remain even in adult brain (Acsady et al., 1998). Although we found that highly motile axonal filopodia are transformed to stable ones during development, we document the continuing existence of a small population of highly motile filopodia at 3 weeks in vitro. This suggests the possibility that a subset of mossy fiber filopodia in the adult brain might show extensive morphological remodeling. A previous EM study (Acsady et al., 1998) found that, in the adult brain, all the mossy fiber filopodia with bulbous terminals had synaptic contacts, suggesting that these filopodia are likely to be stable. At the same time, this study found a subset of filopodia are devoid of bulbous terminals, but these filopodia, unfortunately, were not further analyzed. These nonbulbous filopodia might represent filopodia that show morphological rearrangement and are involved in functional plasticity in mossy fiber-CA3 interneuron synapses. In particular, the dentate gyrus is one of the regions in the CNS where adult neurogenesis occurs and newly generated granule cells extend mossy fiber axons (Gage, 2000). These newly generated neurons are likely to follow developmental stages similar to that in early postnatal period, and their axonal filopodia might show morphological rearrangement comparable to younger neurons during development. If this is the case, the regulation of filopodial motility and synapse formation by KA receptor activation might be important even in the adult life. Since these newborn neurons in the adult are postulated to have a role in learning and memory (Kempermann, 2002), the regulation of filopodia motility by KA receptor activation might be important in these processes.

Implications for Motility of Dendritic Protrusions

Similar to mossy fiber filopodial extensions, the motility of dendritic spines and dendritic filopodia could be affected in opposite directions by glutamate receptor activation. NMDA receptor activation induces the formation of dendritic spines/filopodia in hippocampus (Engert and Bonhoeffer, 1999; Fiala et al., 2002; Maletic-Savatic et al., 1999). In addition, the motility of small dendritic protrusion in retinal ganglion cells is inhibited by antagonists of NMDA and AMPA/KA receptors (Wong et al., 2000). In contrast, stimulation with AMPA, an agonist of AMPA/KA receptor, inhibits spine motility in hippocampal neurons (Fischer et al., 2000). Thus, glutamate receptor activation regulates spine motility in seemingly opposite directions. This paradox could be solved if glutamate had different effects on spine motility at two different stages during synapse formation. First, like in the mossy fiber filopodia, glutamate might induce motility of dendritic spines/filopodia to facilitate finding presynaptic partners. Then, in more mature cells, glutamate could inhibit motility to stabilize synaptic connections. Thus, a similar two-step model could apply to postsynaptic protrusions.

Experimental Procedures

Slice Culture

Hippocampal slices (300 μm thick) were prepared from postnatal day 1 (P1) mice in sterile conditions. Slices were mounted on 0.4 μm culture inserts (Millipore) and incubated (5% CO_2, 37°C) with culture medium, 100 ml of which is composed of 50 ml basal Eagle's medium, 25 ml Hank's balanced salt solution, 1.0 ml HEPES, 100× Pen-Strep (GIBCO), 25 ml horse serum (Hyclone), and 0.65 g dextrose. Hippocampal slice cultures were transfected using the Helios Gene Gun System (Bio-Rad, CA). Plasmid (pEGFP-C1, Clontech) was precipitated onto gold microcarrier particles (1 μm diameter). The DNA loading ratio was 8 μg/mg gold. Culture slices were transfected at 8–20 days in vitro (DIV) and returned to the incubator for 2–6 days posttransfection.

Imaging

Images were acquired with a custom-made two-photon microscope (Majewska et al., 2000) using Fluoview (Olympus) software or an UltraView confocal system (Perkin-Elmers). With a two-photon microscope, images of mossy fiber terminals were acquired at 10× (40× objective) or 6.5× (60× objective) digital zoom, resulting in a nominal spatial resolution of 20 pixels/μm, using 790–850 nm excitation. With an Ultraview confocal system, images were acquired with 100× objective (1.0 NA), resulting in a nominal spatial resolution of 15.2 pixels/μm, using 488 nm excitation. Mossy fibers were identified by the position of cell body and the fiber itself and the characteristic swelling of mossy terminals. Terminals were chosen randomly in the CA3 region. For time-lapse movies, images were taken at 30 s interval over 15 min, and single movies were composed of 30 frames. At each time point, 4–9 focal planes 1 μm apart (two-photon) or 10–20 focal planes 0.6 μm apart (Ultraview confocal) were scanned; these were later projected into a single image. For acute slices, imaging was performed at 5 min intervals.

Analysis

To measure motility of filopodial extensions, the length of filopodia was measured in each frame, the absolute values of length change between consecutive frames were summed over the entire period of image acquisition, and this total length change was divided by time. However, in two-photon microscopy, fluctuations in length of less than 0.3 μm were removed from the analysis to avoid noise arising from movement of the specimens. This threshold was set to 0.15 μm for data obtained with a confocal to compensate for the better spatial resolution of the instrument. Image processing and analysis were performed with custom-written macros using NIH-Image.

Immunocytochemistry

Slices imaged with two-photon microscopy were fixed with 4% paraformaldehyde in phosphate buffer (PB) overnight. Slices were resliced into 50–75 μm thickness. These slices were permeabilized by 1% TritonX 100, blocked by 10% normal donkey serum, incubated by primary antibody (1:1000 dilution; anti-parvalbumin and calretinin, Swant, Switzerland; anti-PSD-95, Chemicon, CA) overnight and then with a Cy3-conjugated secondary antibody (1:100 dilution; Jackson Immuno Research, PA). Images were taken from focal planes 0.5 μm apart using confocal microscope (FV500 IX81, Olympus, NY) equipped with 60× objective (0.9 NA) and Ar (488 nm) and HeNe (543 nm) laser.

Electron Microscopy

Slices imaged with two-photon microscopy were fixed with 5% glutaraldehyde in PB for 1 hr. Slices were embedded in 3% agar and resectioned into 75 μm thick with a vibratome. After locating the sections with the imaged terminals, they were immunostained with rabbit anti-GFP antibody (Chemicon, CA) overnight at 4°C and then with peroxidase-conjugated goat anti-rabbit IgG antibody (Chemicon, CA) for 1 hr at room temperature. After developing with DAB, the sections were postfixed in 1% osmium tetroxide in PB, dehydrated, and then infiltrated with Epox 812 resin (Fullam, NY), placed flat in resin between two plastic slides, and polymerized in the oven at 60°C. After polymerization, plastic slides were separated and the imaged areas of interest were then cut out, mounted on a blank block, and sectioned to 10 μm for examination under phase contrast. The 10 μm sections with the imaged areas of interest were then remounted on a blank block, thin sectioned, and examined in the electron microscope (JEOL 1200EX). Imaged mossy terminals with filopodia were reconstructed from serial thin sections. After

comparison of EM-reconstructed micrographs and two-photon serial images, eight filopodia were identified and retrieved for analysis. Additionally, five filopodia, which were not imaged, were reconstructed. The perimeter of filopodial tips that was not apposed to processes in the neuropil was measured by three different observers and average values were used for analysis.

Acknowledgments

We thank Dr. Juan Lerma for providing GYKI53655 and members of our laboratories for comments. Supported by the National Eye Institute (EY13237), NINDS NS16951 (C.A.M.), the New York STAR Center for High Resolution Imaging of Functional Neural Circuits, and the John Merck Fund. Custom-written alignment software is freely available from the authors.

Received: January 15, 2003
Revised: April 11, 2003
Accepted: April 25, 2003
Published: June 4, 2003

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