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The Rac1-GEF Tiam1 Couples the NMDA Receptor to the Activity-Dependent Development of Dendritic Arbors and Spines

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Summary

NMDA-type glutamate receptors play a critical role in the activity-dependent development and structural remodeling of dendritic arbors and spines. However, the molecular mechanisms that link NMDA receptor activation to changes in dendritic morphology remain unclear. We report that the Rac1-GEF Tiam1 is present in dendrites and spines and is required for their development. Tiam1 interacts with the NMDA receptor and is phosphorylated in a calcium-dependent manner in response to NMDA receptor stimulation. Blockade of Tiam1 function with RNAi and dominant interfering mutants of Tiam1 suggests that Tiam1 mediates effects of the NMDA receptor on dendritic development by inducing Rac1-dependent actin remodeling and protein synthesis. Taken together, these findings define a molecular mechanism by which NMDA receptor signaling controls the growth and morphology of dendritic arbors and spines.

Introduction

Neuronal activity exerts profound effects on the development and structural plasticity of dendritic arbors and spines, the primary sites of excitatory synaptic transmission in the brain (Nikonenko et al., 2002; Wong and Ghosh, 2002). These effects are largely mediated by the N-methyl-D-aspartate (NMDA)-type glutamate receptor, a calcium-permeable ion channel that plays a central role in synaptic development and plasticity (Cull-Candy et al., 2001). Calcium influx through the NMDA receptor activates a number of signaling pathways that have been implicated in the regulation of cytoskeletal dynamics, the promotion of local translation of specific mRNAs, and/or the induction of gene expression (Guo et al., 2001). However, it is not known which of these NMDA receptor-activated signaling pathways mediates NMDA receptor-dependent dendritic growth, spine morphogenesis, and synapse development.

Since the development and structural remodeling of dendrites and spines depends on actin cytoskeletal reorganization (Luo, 2002), it is possible that NMDA receptors regulate dendritic growth and spine morphogenesis by modulating the activity of regulators of the actin cytoskeleton, notably Rho family GTPases. Consistent with this idea, optic nerve stimulation has been shown to alter Rho GTPase activity in a glutamate receptor-dependent manner (Li et al., 2002). Rho GTPases, including Rac1, RhoA, and Cdc42, regulate actin dynamics as well as other cellular processes by functioning as molecular switches, cycling between an active GTP bound state and an inactive GDP bound state. When bound to GTP, Rho GTPases undergo a conformational change that enables them to interact with downstream target molecules and transmit their signals. The activation state of Rho GTPases is controlled by guanine nucleotide exchange factors (GEFs), which stimulate Rho GTPases by catalyzing the exchange of GDP for GTP (Schmidt and Hall, 2002), and GTPase-activating proteins (GAPs), which inhibit Rho GTPases by increasing GTP hydrolysis (Moon and Zheng, 2003). Activation of Rac1 promotes actin polymerization, resulting in the addition and stabilization of dendritic branches (Li et al., 2000) as well as the development and maintenance of spines (Nakayama et al., 2000). By contrast, RhoA activation inhibits dendritic branch extension (Li et al., 2000) and spine formation (Nakayama et al., 2000). While Rho GTPases appear to be required for activity-induced dendritic growth (Sin et al., 2002), it is not clear whether activation of Rac1 or inhibition of RhoA is critical for NMDA receptor-dependent spine morphogenesis, and the mechanism by which NMDA receptors regulate Rho GTPase activity is unknown.

We hypothesized that NMDA receptor activation promotes cytoskeletal remodeling as well as other events important for dendritic arbor growth and spine morphogenesis by stimulating the activity of a Rho family GEF, which in turn activates Rac1. To identify a Rac1-GEF that might regulate dendritic branch and/or spine development in response to NMDA receptor activation, we have begun to examine the expression profiles and the subcellular localization of the known mammalian Rac1-GEFs. This approach led us to consider the role of the Rac1-specific GEF Tiam1 in NMDA receptor-dependent dendrite and spine development. Tiam1 is expressed at high levels in the developing brain, and its expression remains high in adult brain regions undergoing synaptic remodeling (Ehler et al., 1997). In *Drosophila*, the Tiam1 homolog Still life (SIF) is localized at synaptic terminals and plays a role in axonal extension and synaptic development (Sone et al., 1997, 2000).

We show here that Tiam1 is present in the dendrites and spines of mammalian neurons and is necessary for proper dendritic arbor growth and spine and synapse development. We demonstrate that Tiam1 interacts with the NMDA receptor and is required for NMDA re-

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ceptor-dependent spine formation. Tiam1 regulates NMDA receptor-dependent spine formation as well as dendritic arbor growth by activating Rac1, which triggers actin cytoskeletal remodeling that is essential for dendrite and spine development. In addition, Tiam1 appears to contribute to NMDA receptor-mediated dendrite and spine development by modulating activity-dependent protein synthesis (Steward and Schuman, 2001), since we find that Tiam1 is required for the glutamate-induced phosphorylation of the serine/threonine kinase Akt and the eukaryotic initiation factor 4E binding protein (4E-BP), two proteins implicated in the regulation of mRNA translation (Hay and Sonenberg, 2004). Taken together, these results indicate that Tiam1 couples the NMDA receptor to the regulation of dendrite and spine morphology by inducing specific Rac1-dependent intracellular signaling pathways that control actin remodeling and mRNA translation.

Results

Tiam1 Localization in Brain

To determine whether Tiam1 plays a role in NMDA receptor-dependent dendritic development, we first examined the localization of Tiam1 in the brain. Immunofluorescence staining of tissue sections revealed that Tiam1 is widely expressed in the adult rat brain. In the cerebral cortex, Tiam1 staining concentrates in the cell bodies of pyramidal neurons and extends out into the dendrites (Figure 1A). In the CA1 region of hippocampus, Tiam1 is found at low levels in the cytoplasm of pyramidal cells, and at high levels in scattered interneurons (Figure 1B). Tiam1 staining is also visible in the neuropil of stratum radiatum (SR), an area rich in synapses (Figure 1B). To determine the subcellular localization of Tiam1, we incubated hippocampal sections immunostained for Tiam1 with DiO, in order to stain the entire plasma membrane of isolated neurons. At high magnification, Tiam1 puncta are seen in close association with dendrites and spines (Figure 1C). In some cases, Tiam1 puncta lay directly over spine heads, suggesting that Tiam1 is present in spines. In other cases, the Tiam1 puncta appear to lie adjacent to dendritic shafts or spine necks, which may correspond to inhibitory axonal terminals. Tiam1 staining is also enriched in the dendrites of neurons from postnatal day 21 (P21) rat brains, as demonstrated by costaining with the dendrite marker MAP2 (Figure 1D).

To determine more rigorously whether Tiam1 concentrates at synapses, we performed immunogold electron microscopy (EM) on adult rat brain and found that, in the SR of CA1 hippocampus, Tiam1 is localized postsynaptically (Figure 1E). Quantitative analysis revealed that the highest density of Tiam1 labeling is at the synaptic junction, closely associated with the postsynaptic density (PSD) (Figure 1F). Tiam1 labeling was also detected, albeit at considerably lower levels, in the presynaptic axon terminal. The presence of Tiam1 within dendrites and spines suggests that Tiam1 may regulate the development and/or function of these structures.

Association of Tiam1 with the NMDA Receptor

Since Tiam1 is localized in the right place to regulate dendrite and spine development, we considered the possibility that Tiam1 might interact with the NMDA receptor, a critical regulator of dendritic development. To address this possibility, HEK 293T cells were transfected with expression plasmids encoding the NR1 and NR2B subunits of the NMDA receptor with control vector or FLAG-tagged Tiam1, and then Tiam1 was immunoprecipitated with an anti-FLAG antibody. As shown in Figure 2A, NR2B was readily detected in anti-FLAG immunoprecipitates from cells expressing FLAG-Tiam1, but not from control vector transfected cells, indicating that Tiam1 can interact with the NMDA receptor in cells.

To further investigate the nature of this interaction, we examined the ability of Tiam1 to associate with individual NMDA receptor subunits. 293T cells were transfected with expression vectors encoding NR1 or NR2B and FLAG-tagged Tiam1 or Kalirin-7, another Rac1-GEF known to regulate spine morphogenesis (Penzes et al., 2001). Tiam1 and Kalirin-7 were immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitates were probed with anti-NR1 or -NR2B antibodies. We found that NR1 coimmunoprecipitates with Tiam1 but not with Kalirin-7 (Figure 2B), indicating that Tiam1 can specifically interact with NR1 in cells in the absence of NR2B. In contrast, we were unable to detect a specific interaction between NR2B and Tiam1 when these proteins were expressed together in the absence of NR1 (data not shown). These results suggest that the interaction between Tiam1 and the NMDA receptor is mediated by NR1. However, we cannot exclude the possibility that NR2B also interacts with Tiam1, but that the interaction is not detected because NR2B is not transported efficiently to the cell surface in the absence of NR1 (Garcia-Gallo et al., 2001; Perez-Otano et al., 2001).

To determine if the interaction between Tiam1 and the NMDA receptor occurs when these proteins are expressed at normal physiological levels, Tiam1 was immunoprecipitated from synaptosomes purified from P15 rat brains. Since NR1 and NR2B interact in neurons, we would expect that if NR1 and Tiam1 also interact, immunoprecipitation of Tiam1 should lead to a coprecipitation of both NR1 and NR2B. Therefore, to detect an interaction between Tiam1 and the NMDA receptor, we probed the Tiam1 immunoprecipitates with anti-NR2B antibodies, since in our experience, anti-NR2B antibodies recognize endogenous NMDA receptors more efficiently than anti-NR1 antibodies. We found that NR2B coimmunoprecipitates with Tiam1 but not with an unrelated protein (ZAP70) (Figure 2C), demonstrating that Tiam1 and the NMDA receptor specifically associate in the brain.

To corroborate our finding that Tiam1 and the NMDA receptor interact, we asked whether Tiam1 and the NMDA receptor colocalize in neurons. NMDA receptor localization and function can be acutely modulated in neurons by the EphB class of receptor tyrosine kinases (Dalva et al., 2000; Grunwald et al., 2001; Henderson et al., 2001; Takasu et al., 2002). Specifically, ephrinB activation of EphBs induces the coclustering of EphB and NMDA receptors, resulting in the tyrosine phosphorylation of the NMDA receptor subunit NR2B and the potentiation of calcium influx through NMDA receptors. To examine whether Tiam1 colocalizes with the NMDA receptor in these clusters in response to EphB

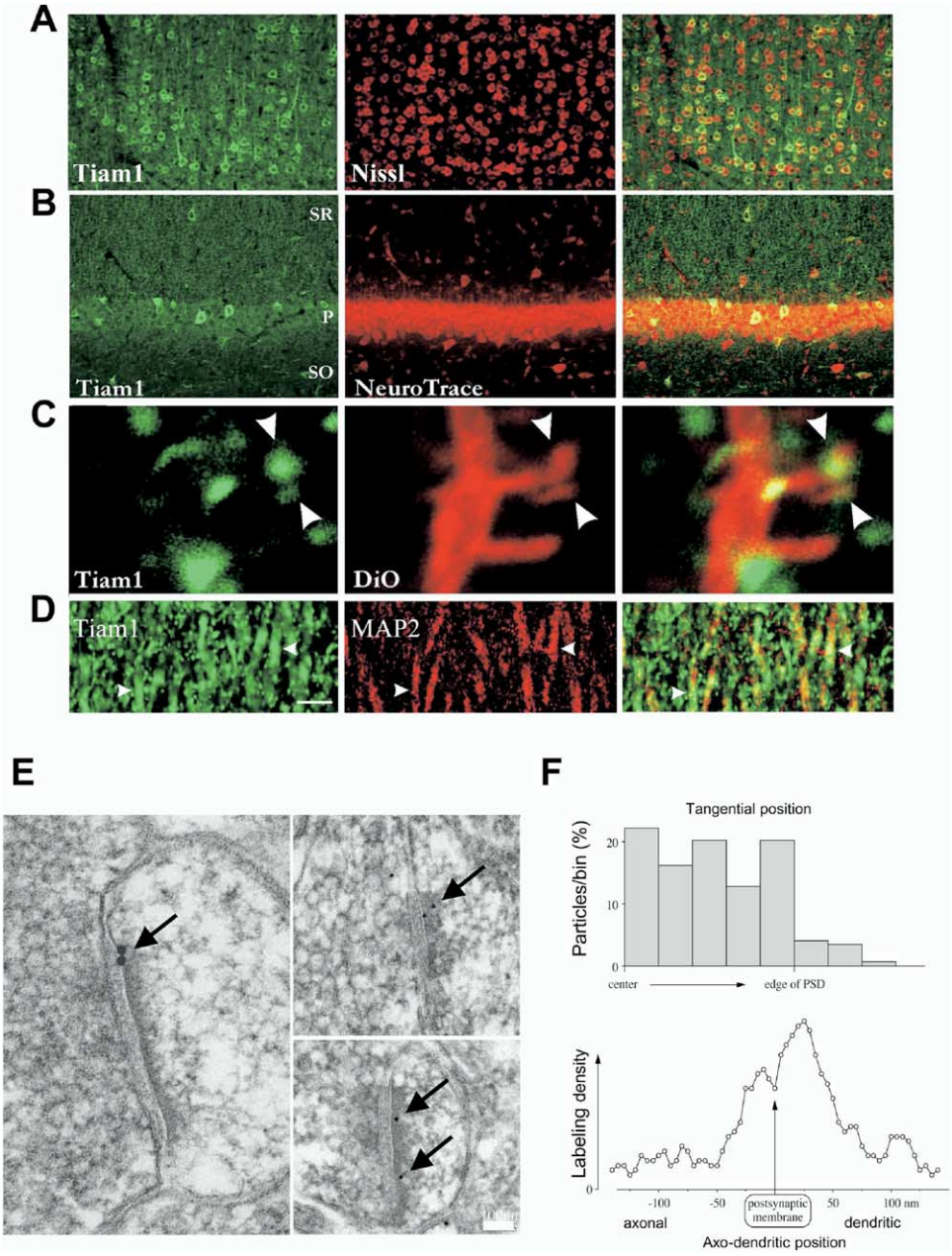

Figure 1. Tiam1 Localization in Rat Brain

(A) Tiam1 localization in the cerebral cortex. Tiam1 staining (green) is present in the somata (defined by Nissl stain; red) of layer V cortical neurons and extends into apical dendrites. Yellow indicates regions of colocalization.

(B) Distribution of Tiam1 in adult CA1. Somata of scattered interneurons are strongly immunopositive for Tiam1, whereas somata of pyramidal cells in the pyramidal cell layer (P) are more weakly stained; staining spares the nucleus. Staining is also visible in the neuropil of stratum radiatum (SR).

(C) Distribution of Tiam1 in relationship to dendritic spines. Section has been treated with DiO (red), allowing visualization of dendritic plasma membrane. Tiam1 puncta appear to lie inside the heads of two spines (arrowheads).

(D) Tiam1 staining in SR of CA1 hippocampus from P21 rat. Apical dendrites (defined by MAP2 staining; red) express high levels of Tiam1 (arrowhead). Bar represents 10 μM.

(E) Postembedding immunogold EM labeling of Tiam1 at asymmetric axospinous synapses from adult rat hippocampus. Arrows point to gold particles coding for Tiam1 at the postsynaptic density (PSD) in dendritic spines; 10 nm gold particles were used. Bar represents 100 nm.

(F) Quantitative analysis of the distribution of Tiam1 immunogold particles at synapses. (Top panel) Histogram shows the distance of gold particles from the center of the PSD in the tangential axis of the synapse (normalized by PSD length); labeling close to the plasma membrane is coextensive with the synaptic specialization. (Bottom panel) Graph shows the axodendritic distribution of gold particles for Tiam1. Particle counts for 5 nm bins were smoothed with 5-point weighted averaging to estimate particle densities. Tiam1 shows the highest density at the synaptic junction. Data were collected from randomly selected fields containing a clearly defined synapse from two rats, including 149 particles within ±150 nm of the PSD.

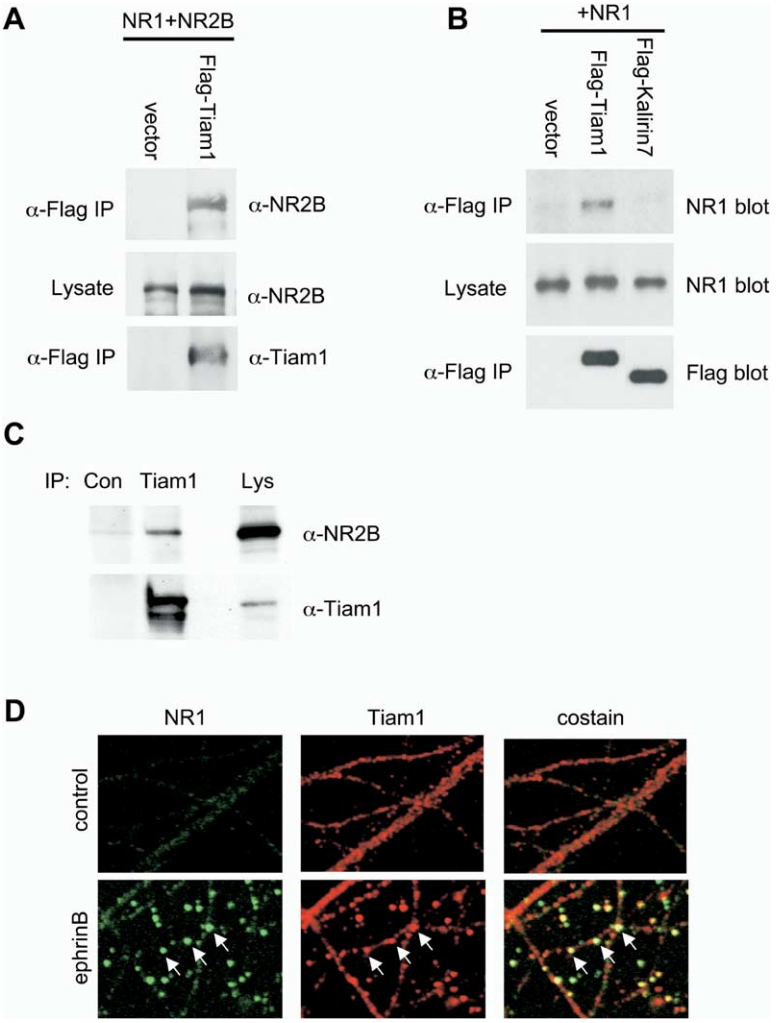

Figure 2. Tiam1 Interacts and Colocalizes with the NMDA Receptor

(A) Coassociation of Tiam1 with the NMDA receptor in 293T cells. Lysates from 293T cells transfected with the NMDA receptor subunits NR1 and NR2B in combination with control vector or FLAG-tagged Tiam1 were immunoprecipitated with anti-FLAG antibodies. Presence of NR2B and Tiam1 in the immunoprecipitates and cell lysates was detected by immunoblotting.

(B) Interaction of Tiam1 with NR1. Extracts of 293T cells transfected with NR1 in combination with control vector, FLAG-Tiam1, or FLAG-Kalirin-7 were immunoprecipitated with anti-FLAG antibodies and then immunoblotted with anti-NR1 antibodies.

(C) Association of Tiam1 and NR2B in P15 rat brain synaptosomes. Lysate from P15 rat synaptosomes was immunoprecipitated with anti-Tiam1 antibodies or with control (anti-ZAP70) antibodies and then immunoblotted with anti-NR2B antibodies. The presence of NR2B and Tiam1 in the synaptosome lysate (Lys) was confirmed by immunoblotting.

(D) Tiam1 colocalizes with the NMDA receptor upon ephrinB stimulation. Hippocampal neurons were treated with aggregated ephrinB1-Fc or Fc for 60 min and then fixed and stained for Tiam1 (red) and NR1 (green). The white arrows indicate Tiam1 and NR1 colocalization (yellow).

activation, we treated embryonic day 18 (E18) rat hippocampal neurons (6 days in vitro [DIV6]) with aggregated ephrinB1-Fc or Fc control protein. Following stimulation with ephrinB1-Fc, the neurons were fixed and costained with antibodies to NR1 and Tiam1 to assess colocalization. We found that ephrinB1 stimulation promotes the clustering of both Tiam1 and NR1 along neurites and that these Tiam1 and NR1 puncta colocalize (Figure 2D). In contrast, Tiam1 and NR1 colocalization was not detected when neurons were subjected to control (Fc) treatment (Figure 2D) or stimulated with ephrinA1-Fc (data not shown). These findings indicate that Tiam1 is recruited to NMDA receptor complexes following EphB activation and suggest that Tiam1 may be involved in NMDA receptor signaling in neurons.

NMDA Receptor Stimulation Induces Tiam1 Phosphorylation and Rac1 Activation

Our observation that Tiam1 and the NMDA receptor interact led us to investigate whether NMDA receptor-dependent signaling activates Tiam1's GEF activity. We found that glutamate stimulation of cortical (Figure 3A) and hippocampal (data not shown) neurons (DIV7) induces a shift in the mobility of Tiam1 on a SDS-PAGE gel, raising the possibility that glutamate induces Tiam1 phosphorylation. To verify that the glutamate-induced Tiam1 shift is due to phosphorylation, we incubated glutamate-stimulated neuronal lysate with alkaline phosphatase in the presence or absence of phosphatase inhibitors. We found that alkaline phosphatase treatment abolished the Tiam1 shift, and this effect was blocked by the addition of phosphatase inhibitors (Figure 3A), indicating that Tiam1 is phosphorylated in response to glutamate stimulation. The glutamate-induced Tiam1 phosphorylation appears to require calcium influx through the NMDA receptor, since both 2-amino-5-phosphonovalerate (APV), a NMDA receptor antagonist, and EGTA, a calcium chelator, block the glutamate-induced Tiam1 mobility shift (Figures 3B and 3C). Consistent with these results, we also found that Tiam1 is phosphorylated in P1 hippocampal neurons (10 DIV) in response to neuronal activity induced by blocking inhibitory synaptic inputs with the $GABA_A$ receptor antagonist bicuculline (data not shown). Taken together, these results indicate that calcium influx through the NMDA receptor induces Tiam1 phosphorylation.

Phosphorylation has previously been reported to modulate Tiam1 GEF activity. For example, the Ca^{2+}/

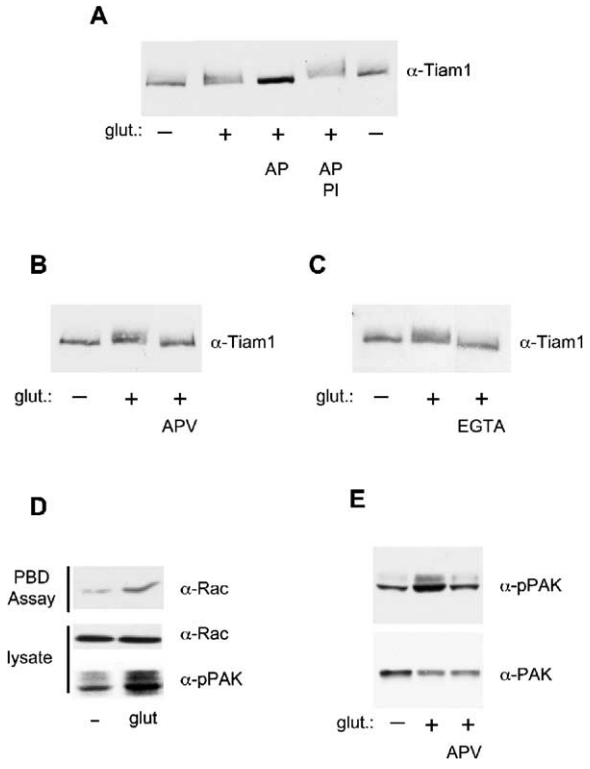

Figure 3. NMDA Receptor Stimulation Induces Tiam1 Phosphorylation and Rac1 Activation

(A) Tiam1 is phosphorylated in response to NMDA receptor stimulation. Cortical neurons pretreated with TTX, CNQX, and nimodipine were stimulated with 50 μM glutamate for 5 min and then lysed and subjected to Western blot analysis using anti-Tiam1 antibodies. Stimulation of neurons with glutamate induces a shift in the mobility of Tiam1 on an SDS-PAGE gel. To verify that the glutamate-induced Tiam1 shift is due to phosphorylation, glutamate-stimulated neuronal lysate was incubated with alkaline phosphatase (AP) in the presence or absence of phosphatase inhibitors (PI).

(B) Glutamate-induced Tiam1 phosphorylation requires the NMDA receptor. To examine the involvement of the NMDA receptor in glutamate-induced Tiam1 phosphorylation, neurons were pretreated with APV (100 μM) for 30 min before glutamate stimulation.

(C) Tiam1 phosphorylation in response to NMDA receptor stimulation is Ca^{2+} dependent. To assess whether glutamate-induced Tiam1 phosphorylation requires Ca^{2+} influx through the NMDA receptor, neurons were preincubated with EGTA (2 mM) for 30 min prior to glutamate stimulation.

(D) Rac1 is activated by glutamate stimulation of neurons. Cortical neurons were stimulated with 50 μM glutamate for 5 min. Rac1 activation was assessed by a Pak-PBD pulldown assay, followed by immunoblotting with anti-Rac1 antibodies. Pak phosphorylation was assessed with an anti-pPak antibody.

(E) Pak phosphorylation is induced by NMDA receptor stimulation. Cortical neurons were stimulated as above in the presence or absence of APV (100 μM/30 min pretreatment). Pak phosphorylation was assessed with an anti-pPak antibody.

calmodulin-dependent protein kinase II (CaMKII) enhances Tiam1's GEF activity toward Rac1 by phosphorylating Tiam1 on serine/threonine residues (Fleming et al., 1999). Since NMDA receptor stimulation induces both CaMKII activation (Lisman et al., 2002) and Tiam1 phosphorylation, we examined whether calcium influx through the NMDA receptor also regulates the activation of the Tiam1 effector protein Rac1. The level of active Rac1 (Rac1-GTP) in neurons was measured with a Pak-PBD binding assay, which utilizes the Rac1 binding domain of Pak as an affinity reagent to precipitate Rac1-GTP from cells (Sander et al., 1998). We found that glutamate stimulation of neurons increases the level of active Rac1 in neurons (Figure 3D). Glutamate also induces the phosphorylation of Pak, a downstream target of Rac1, and this phosphorylation is NMDA receptor dependent, since it is blocked by pretreatment with APV (Figures 3D and 3E). These findings are consistent with previous reports demonstrating that optic nerve stimulation activates Rac1 in a glutamate receptor-dependent manner (Li et al., 2002; Sin et al., 2002) and suggest that NMDA receptor signaling induces the phosphorylation of Tiam1, thereby stimulating local Rac1 activation.

Role for Tiam1 in the Development of Dendrites and Spines/Synapses

Rac1 plays a central role in the regulation of dendritic branch and spine morphology. The evidence that the Rac1-GEF Tiam1 is present in dendrites and spines and forms a complex with the NMDA receptor suggests that, by activating Rac1, Tiam1 may mediate some of the effects of the NMDA receptor on dendritic development and/or spine morphogenesis. To determine whether Tiam1 is required for these processes, we employed the plasmid-based pSUPER RNA interference (RNAi) system (Brummelkamp et al., 2002) to knock down the expression of Tiam1 in cultured neurons. We identified a 19 bp siRNA sequence (pSUPER-Tiam1 RNAi) that specifically reduces Tiam1 protein in cultured hippocampal (Figure 4A) and cortical (Figure 7B) neurons without affecting the levels of other cellular proteins. This decrease in Tiam1 expression was not detected in neurons expressing empty pSUPER vector or another pSUPER RNAi construct (pSUPER-MEF2D RNAi), which specifically knocks down MEF2D (Figure 4A), a transcription factor expressed in neurons.

To assess the involvement of Tiam1 in dendritic branch development, E18 dissociated hippocampal neurons (DIV9) were transfected with pSUPER, pSUPER-Tiam1 RNAi, or pSUPER-MEF2D RNAi in combination with an enhanced green fluorescent protein (eGFP) expression vector. To exclude the possibility that any effects seen with Tiam1 RNAi were due to apoptosis, we included a plasmid encoding the antiapoptotic protein Bcl-X_L in some of the transfections. While Bcl-X_L is known to inhibit cell death, it does not affect dendritic growth (Gaudilliere et al., 2004). Neurons were fixed and imaged 12 days after transfection (DIV21). The effects of Tiam1 knockdown on dendritic growth and branching were then determined using Sholl analysis, which quantifies the number of times dendrites from a neuron cross concentric circles of increasing diameter (Sholl, 1953). Using this analysis, we found that reducing Tiam1 levels in neurons results in a simplification of their dendritic tree (Figure 4B). Similar results were seen in neurons expressing pSUPER-Tiam1 RNAi in the presence or absence of Bcl-X_L (data not shown). The decrease in dendritic complexity caused by Tiam1 RNAi appears to be specific and is not due to the activation

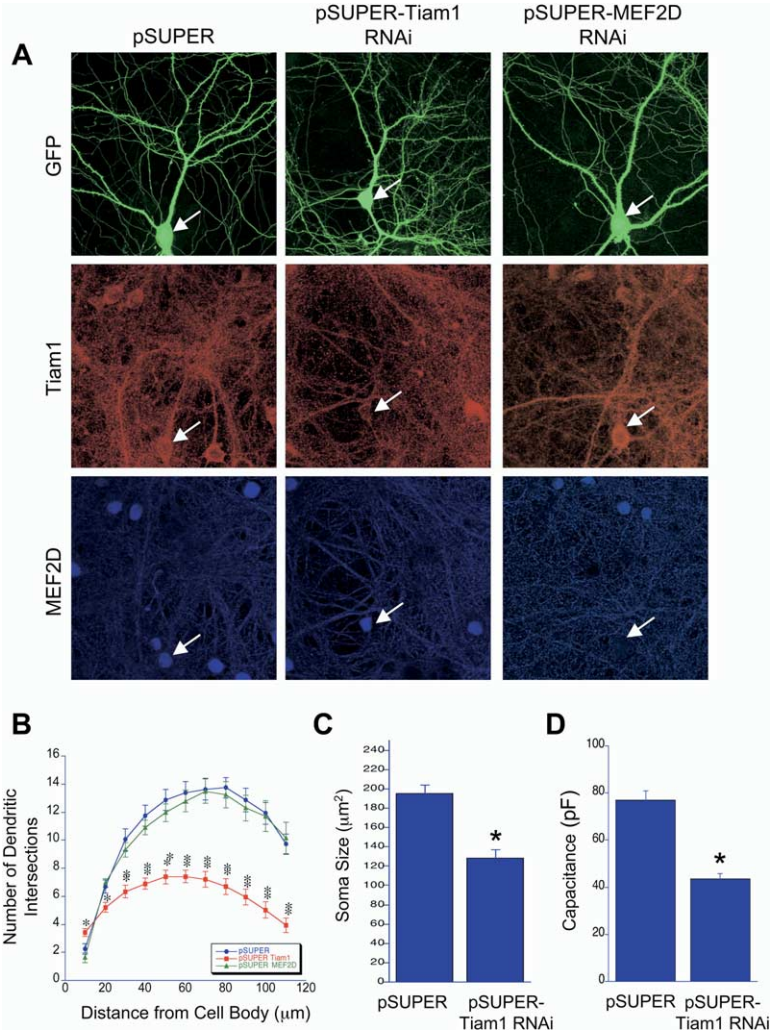

Figure 4. RNAi Knockdown of Tiam1 Expression Causes a Simplification of the Dendritic Tree

(A) Reduced expression of Tiam1 in hippocampal neurons using RNAi. Dissociated hippocampal neurons were transfected (DIV9) with eGFP and Bcl-X_L together with pSUPER, pSUPER-Tiam1 RNAi, or pSUPER-MEF2D RNAi. At DIV21, neurons were fixed and subjected to immunofluorescence. Tiam1 expression was specifically reduced in neurons transfected with pSUPER-Tiam1 RNAi (arrow), but not in neighboring untransfected cells. In contrast, the expression of another neuronal protein, MEF2D, was not affected by pSUPER-Tiam1 RNAi, nor were Tiam1 levels affected by pSUPER or pSUPER-MEF2D RNAi. (B) Tiam1 knockdown results in a simplification of the dendritic arbor of hippocampal neurons. Sholl analysis of neurons expressing pSUPER, pSUPER-Tiam1 RNAi, or pSUPER-MEF2D RNAi revealed that reducing Tiam1 levels with RNAi causes a significant decrease in dendritic complexity (*$p < 0.05$; **$p < 0.001$). (C) Hippocampal neurons transfected with eGFP and Bcl-X_L together with pSUPER or pSUPER-Tiam1 RNAi were fixed at 21 DIV, and the cell soma area was outlined and measured (*$p < 0.001$). (D) Capacitance measurements of hippocampal neurons. Neurons were transfected with control or pSUPER-Tiam1 RNAi constructs at 9 DIV, and capacitance was measured at 19 DIV. A decrease in capacitance was seen in neurons transfected with pSUPER-Tiam1 RNAi (*$p < 0.001$). All error bars are standard error of the mean (SEM).

of the RNAi machinery per se, since expression of pSUPER-MEF2D RNAi did not result in dendritic arbor simplification (Figure 4B). This result indicates that Tiam1 is critical for dendritic development and likely plays a role in dendritic growth, branching, and/or maintenance.

In addition to causing a reduction in dendritic arbor complexity, we also found that knockdown of Tiam1 expression affects neuronal soma size (Figure S1A in the Supplemental Data available with this article online). We assessed soma size by measuring soma footprint area of Tiam1 RNAi-treated cells compared to control-transfected neurons. We found that Tiam1 RNAi causes a significant decrease in cell soma size (Figure 4C). Consistent with this result, we also observed a decrease in membrane capacitance in cells transfected with Tiam1 RNAi as measured by whole-cell voltage clamp at the cell body (Figure 4D). Taken together, these results indicate that Tiam1 regulates cell size. It is possible that Tiam1 utilizes a common mechanism to promote the growth of dendritic arbors and neuronal soma size.

An important aspect of dendritic development is the formation and morphogenesis of dendritic spines. Since Tiam1 is present in spines (Figure 1D), we also asked whether Tiam1 regulates spine morphogenesis. We found that while the dendrites of pSUPER-expressing neurons were studded with spines, the dendrites of neurons expressing pSUPER-Tiam1 RNAi had significantly fewer spines per micron (Figure 5A). Quantification revealed that, while control neurons transfected with pSUPER or pSUPER-MEF2D RNAi had a similar spine density (0.79 ± 0.02 and 0.79 ± 0.04 spines/μm, respectively), neurons transfected with pSUPER-Tiam1 RNAi showed a significant reduction in spine density (0.52 ± 0.02 spines/μm; $p < 0.001$) (Figure 5B). To further examine the effect of knocking down Tiam1 expression on spine morphogenesis, we measured the length of the remaining spines. We found that the spines from neurons transfected with pSUPER-Tiam1 RNAi were on average longer and more filopodia-like than the spines from pSUPER-transfected neurons (Figure S1B). The finding that Tiam1 knockdown reduces spine density and increases spine length is consistent with a blockade in Rac1 activity (Nakayama et al., 2000; Tashiro and Yuste, 2004). These results indicate that a decrease in

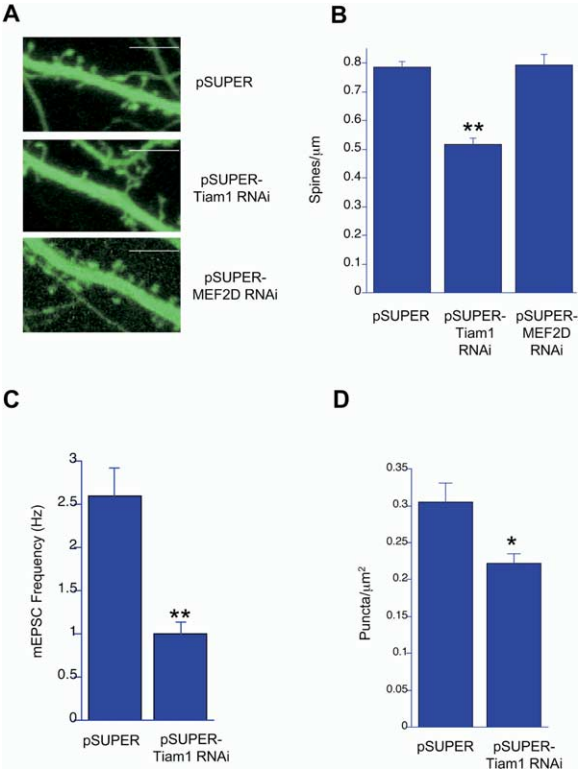

Figure 5. Reduced Tiam1 Expression Results in Decreased Spine and Synapse Density

(A) Knockdown of Tiam1 expression results in a decreased density of spines. Hippocampal neurons transfected with pSUPER, pSUPER-Tiam1 RNAi, or pSUPER-MEF2D RNAi in combination with eGFP and Bcl-X_L were fixed and subjected to immunofluorescence at 21 DIV. Hippocampal neurons transfected with pSUPER-Tiam1 RNAi had fewer spines compared to neurons transfected with pSUPER or pSUPER-MEF2D RNAi. (B) Quantification of the effect of decreased Tiam1 expression on spine density (**$p < 0.001$). (C) Tiam1 knockdown results in a decrease in mEPSC frequency (**$p < 0.001$). (D) Tiam1 is required for synapse development. The density of synapses of hippocampal neurons expressing pSUPER-Tiam1 RNAi was significantly reduced compared to control neurons expressing pSUPER (*$p < 0.001$). All error bars are SEM.

Tiam1 expression perturbs spine development, raising the possibility that Tiam1 is required for Rac-dependent spine development and/or maintenance.

Since spines are the postsynaptic sites of excitatory synapses, we examined whether the decrease in spine density caused by Tiam1 RNAi corresponds to a decrease in excitatory synapse density. To assess synapse number, we recorded spontaneous miniature excitatory postsynaptic currents (mEPSCs) from hippocampal neurons transfected with pSUPER or pSUPER-Tiam1 RNAi. mEPSCs are caused by the spontaneous release of a quantum of neurotransmitter from a presynaptic terminal. Thus, the underlying cause of a change in mEPSC frequency could be a change in synapse number. We found that the mEPSC frequency of neurons expressing pSUPER-Tiam1 RNAi was 62% less than control neurons (Figure 5C; $p < 0.001$), suggesting that Tiam1 knockdown results in fewer synapses. However, we cannot exclude the possibility that the decrease in mEPSC frequency caused by Tiam1 RNAi may be due in part to a simplification of the dendritic arbor (Figure 4B), which could change total synapse number, or reflect a change in presynaptic release probability. To address these issues, we directly measured synapse density using an independent, immunocytochemical approach. To identify synapses, cultured hippocampal neurons expressing eGFP in combination with pSUPER or pSUPER-Tiam1 RNAi were fixed and double labeled with antibodies to the postsynaptic marker PSD-95 and the presynaptic marker synapsin. We then quantified the density of synapses on transfected cells by counting the number of colocalized PSD-95 and synapsin puncta per area of dendrite. In agreement with the decrease in mEPSC frequency, we found that neurons transfected with pSUPER-Tiam1 RNAi had 29% fewer synapses per area of dendrite than control neurons (Figure 5D; $p < 0.001$). Taken together, these results suggest that Tiam1 is required for proper synapse development.

To confirm a role for Tiam1 in spine development using an independent approach, we employed either of two dominant interfering forms of Tiam1 to inhibit Tiam1 function. The PHn-CC-Ex domain of Tiam1 has previously been shown to act in a specific dominant-negative manner (Kawauchi et al., 2003; Stam et al., 1997), most likely by binding to Tiam1-interacting proteins and thereby blocking the recruitment of endogenous Tiam1 to the plasma membrane where Tiam1 is active (Michiels et al., 1997). Consistent with our RNAi studies, we found that dissociated hippocampal neurons expressing the PHn-CC-Ex domain had significantly fewer spines per micron compared to control vector-transfected neurons (0.31 ± 0.03 versus 0.52 ± 0.02 spines/μm; $p < 0.001$) (Figures 6A and 6B). Similar results were also obtained when the Tiam1 PHn-CC-Ex domain was expressed in pyramidal neurons within the context of an organotypic slice culture obtained from P7 rat hippocampus (Figure 6C). Neurons expressing the Tiam1 PHn-CC-Ex domain exhibited a marked reduction in spine density compared to control-transfected neurons (0.34 ± 0.07 versus 0.62 ± 0.07 spines/μm; $p < 0.05$).

As Tiam1 is a multidomain protein that possesses functions in addition to its ability to activate Rac1, we also generated a form of Tiam1 (Tiam1QK) that has specific mutations in the DH-PH domain that render it inactive as a Rac1-GEF (Figure S2A). If overexpression of this Tiam1QK mutant in neurons were to lead to a decrease in spine density, this would suggest that Tiam1 promotes spine development by activating Rac1. We found that expression of the Tiam1QK mutant in hippocampal neurons from P7 organotypic slice cultures causes a marked reduction in dendritic spine density compared to control-transfected neurons (0.09 ± 0.05 versus 0.62 ± 0.07 spines/μm; $p < 0.001$) (Figure 6C), suggesting that Tiam1 regulates spine development by activating Rac1.

In contrast to the RNAi and dominant-negative mutant experiments that demonstrate that blockade of Tiam1 function causes a decrease in spine density, we found that overexpression of wild-type Tiam1 in dissociated hippocampal neurons leads to a small but statis-

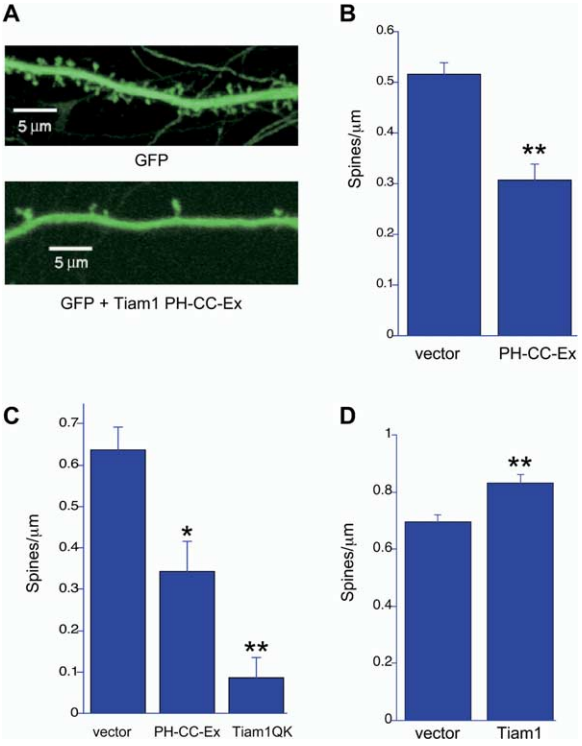

Figure 6. Effect of Overexpression of Tiam1 Mutants on Spine Density

(A) The Tiam1 PHn-CC-Ex domain inhibits dendritic spine development in dissociated hippocampal neurons. Hippocampal neurons were transfected with eGFP in combination with control vector or myc-tagged Tiam1 PHn-CC-Ex domain. At 21 DIV, neurons were fixed, and the spines of transfected neurons were imaged using a laser-scanning confocal microscope. (B) Quantification of the effects of the Tiam1 PHn-CC-Ex domain on dendritic spine density in cultured neurons (**p < 0.001). (C) The Tiam1 PHn-CC-Ex domain and the Tiam1QK mutant inhibit spine development in organotypic hippocampal slice cultures. Hippocampal slices prepared from P7 rat pups (2 DIV) were transfected with eGFP in combination with control vector, the Tiam1 PHn-CC-Ex domain, or the Tiam1QK mutant using biolistic transfection, and transfected pyramidal neurons were imaged 6 days later (*p < 0.05; **p < 0.001). (D) Tiam1 promotes dendritic spine development of dissociated hippocampal neurons. Hippocampal neurons were transfected with eGFP in combination with control vector or myc-tagged Tiam1. At 21 DIV, neurons were fixed, and the spines of transfected neurons were imaged using a laser-scanning confocal microscope (**p < 0.001). All error bars are SEM.

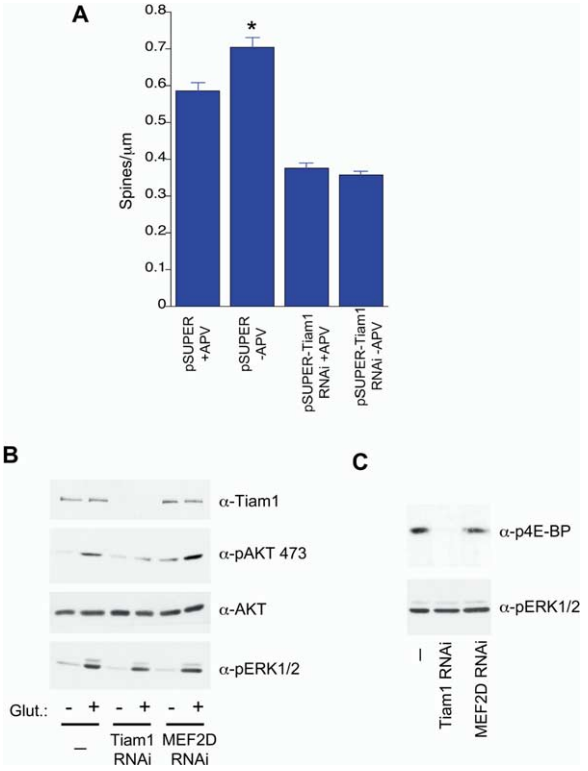

Figure 7. Tiam1 Is Required for NMDA Receptor-Dependent Spine Growth and Intracellular Signaling

(A) NMDA receptor-dependent dendritic spine growth is blocked by Tiam1 RNAi. Cortical neurons transfected at 9 DIV were chronically treated with APV (200 μM) starting at 10 DIV. At 21 DIV, medium was replaced with ACSF for 30 min in the presence or absence of APV (200 μM) to allow for endogenous glutamate release. After a 2 hr recovery period, the neurons were fixed and analyzed (*p < 0.001). Error bars are SEM.

(B and C) Cortical neurons were infected at 1 DIV with Tiam1 RNAi lentivirus, MEF2D RNAi lentivirus, or no virus. On DIV7, cortical neurons that had been pretreated with TTX, CNQX, and nimodipine were stimulated with 50 μM glutamate for 5 min. Neurons were lysed, and Western analysis was performed with antibodies raised against Tiam1, Akt, pAkt (Ser473), and pERK1/2 (Thr202/Tyr204) (B) and p4E-BP (Thr37/46) (C).

tically significant increase in spine density compared to control vector-transfected neurons (0.83 ± 0.03 versus 0.69 ± 0.03; p < 0.001) (Figure 6D). These findings suggest a role for Tiam1 in spine development and raise the possibility that, in some contexts, Tiam1 may promote new spine formation.

Tiam1 Is Required for NMDA Receptor-Dependent Spine Formation and Intracellular Signaling

To examine directly the role of Tiam1 in NMDA receptor-induced spine growth, we utilized a previously developed culture paradigm for acute NMDA receptor-induced spinogenesis (Goldin et al., 2001; Lin et al., 2004). In this system, high-density cortical neurons are grown in the presence of the NMDA receptor antagonist APV and then exposed briefly to a medium that favors NMDA receptor activation by spontaneous synaptic activity. Using this protocol, we found that, following NMDA receptor stimulation, neurons transfected with pSUPER showed a significant increase in dendritic spine density (Figure 7A). To examine whether Tiam1 is required for this NMDA receptor-dependent increase in spine density, we repeated the stimulation protocol with cortical neurons transfected with pSUPER-Tiam1 RNAi. We found that, prior to stimulation, cortical neurons expressing Tiam1 RNAi possessed fewer spines per micron than control neurons (Figure 7A). Importantly, NMDA receptor stimulation of these cells failed to induce an increase in spine density (Figure 7A), indicating that Tiam1 plays a role in NMDA receptor-mediated spine development.

Since Tiam1 is a Rac1-specific GEF, it likely regulates

NMDA receptor-dependent spine formation as well as dendritic arbor growth by activating Rac1, thereby triggering the actin cytoskeletal remodeling essential for spine and dendrite development. Support for this possibility is provided by the evidence that expression of a dominant-negative mutant of Tiam1 that lacks GEF activity blocks proper spine development (Figure 6C). To gain further insight into Tiam1's role in NMDA receptor-dependent dendritic development, we sought to determine the importance of Tiam1 for other downstream signaling events induced by NMDA receptor stimulation. Calcium influx through NMDA receptors activates multiple pathways that regulate a variety of cellular processes important to dendritic development, including AMPA receptor activity and trafficking, cytoskeletal dynamics, mRNA translation, and gene expression (Bredt and Nicoll, 2003; Nikonenko et al., 2002; Steward and Schuman, 2003; West et al., 2001). For instance, NMDA receptor stimulation has been shown to activate the Phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Perkinton et al., 2002), which controls cell growth and survival as well as actin cytoskeletal remodeling (Cantrell, 2001). NMDA receptor stimulation also leads to the activation of the Ras/ERK pathway that promotes gene transcription by inducing the phosphorylation/activation of transcription factors such as CREB (West et al., 2002). To examine Tiam1's role in these NMDA receptor-dependent events, we used a lentiviral-based RNAi system that allowed us to specifically knock down Tiam1 expression in the majority of cortical neurons in a dissociated culture, without affecting the expression levels of other proteins (Figure 7B). Cortical neurons were infected with Tiam1 siRNA-expressing lentivirus, MEF2D siRNA-expressing lentivirus (as a control), or no virus on DIV1 and then cultured for 6 days to allow for protein knockdown. On DIV7, the cultures were briefly exposed to 50 μM glutamate to stimulate NMDA receptors and then harvested. Western blotting analysis revealed that the phosphorylation of the serine/threonine kinase Akt on serine 473 (Figure 7B), a phosphorylation site that correlates with Akt activation (Alessi et al., 1996), was specifically abrogated in neurons in which Tiam1 protein was knocked down (Figure 7B). In contrast, reduced Tiam1 expression did not appear to significantly affect NMDA receptor-dependent ERK1/2 phosphorylation (Figure 7B) or IGF-1-induced Akt phosphorylation (Figure S2B), suggesting that the effect of Tiam1 RNAi on NMDA receptor-dependent Akt phosphorylation does not reflect a general defect in signal transduction or a failure of the Tiam1 RNAi-expressing cells to flux Ca^{2+} in response to glutamate treatment.

The ability of Tiam1 to activate the PI3K/Akt pathway downstream of NMDA receptors may enable Tiam1 to induce Rac1-dependent actin remodeling, since the PI3K/Akt pathway has been implicated in actin cytoskeletal regulation (Merlot and Firtel, 2003). In addition to actin remodeling, the PI3K/Akt pathway is known to play a critical role in regulating mRNA translation (Kozma and Thomas, 2002). The PI3K/Akt pathway stimulates protein synthesis by inducing the activation of mammalian Target of Rapamycin (mTOR), which phosphorylates and inactivates the repressor of mRNA translation, 4E-BP. Given that Tiam1 is required for the glutamate-induced phosphorylation/activation of Akt, we examined whether knockdown of Tiam1 expression affects 4E-BP phosphorylation. We found that 4E-BP phosphorylation was specifically blocked in neurons with reduced Tiam1 expression (Figure 7C), suggesting that Tiam1 plays a role in regulating NMDA receptor-dependent mRNA translation. These results indicate that Tiam1 plays an unexpected role in the NMDA receptor-dependent activation of the PI3K-Akt pathway and may thereby regulate dendrite and spine development by inducing both actin remodeling and protein synthesis. As NMDA receptors are localized within spines, Ca^{2+} influx through NMDA receptors may trigger the local activation of Tiam1, leading to Rac1 activation and actin remodeling that is required for spine growth. In addition, Ca^{2+} influx may induce Akt and 4E-BP phosphorylation by a Tiam1-Rac1-dependent mechanism, resulting in a local increase in protein synthesis that similarly contributes to spine growth.

Discussion

The NMDA receptor plays a critical role in the activity-dependent development and plasticity of dendritic arbors, spines, and synapses (Nikonenko et al., 2002; Wong and Ghosh, 2002). However, relatively little is known about the underlying mechanisms that link NMDA receptor activation to the growth and remodeling of dendrites and spines. In this study, we identify the Rac1-GEF Tiam1 as a crucial mediator of NMDA receptor-dependent dendritic development. We show that Tiam1 is present in dendrites and spines and is required for dendritic arbor growth and spine/synapse development. Tiam1 interacts with the NMDA receptor and is phosphorylated in a calcium-dependent manner following NMDA receptor activation. By activating Rac1 in response to NMDA receptor stimulation, Tiam1 may promote actin cytoskeletal remodeling as well as other processes necessary for dendritic arbor growth, spine morphogenesis and synapse development and plasticity (Bonhoeffer and Yuste, 2002; Van Aelst and Cline, 2004). This possibility is supported by the finding that knocking down Tiam1 expression with RNAi blocks NMDA receptor-induced spine outgrowth, indicating that Tiam1 is required for NMDA receptor-mediated spine formation.

The precise mechanism that couples NMDA receptor stimulation to Tiam1-dependent Rac1 activation remains to be determined. Ca^{2+} entry through the NMDA receptor triggers multiple signaling pathways that may contribute to Tiam1 regulation (Figure 8). For instance, members of the CaMK family are activated in response to NMDA receptor stimulation (Lisman et al., 2002). Since CaMKII has been shown to phosphorylate Tiam1 and stimulate Tiam1-dependent Rac1 activation (Fleming et al., 1999), we examined whether the NMDA receptor regulates Tiam1 activity via phosphorylation. Our finding that Ca^{2+} influx through the NMDA receptor induces both Tiam1 phosphorylation and Rac1 activation suggests that the NMDA receptor activates Tiam1 by triggering its phosphorylation by a Ca^{2+}-dependent kinase. Initial studies to identify Ca^{2+}-dependent Tiam1 phosphorylation sites using mass spectrometry indicate that Tiam1 is phosphorylated on multiple sites

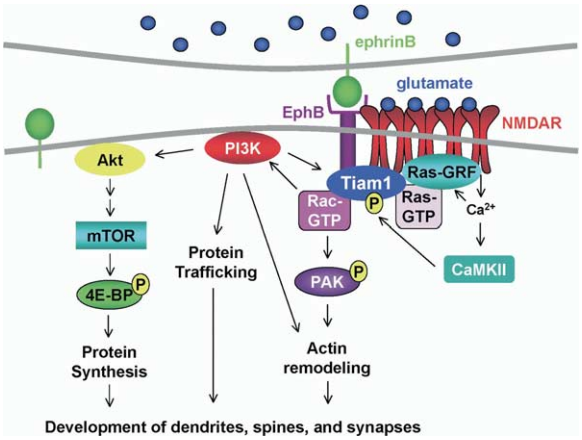

Figure 8. Model Depicting the Potential Role of Tiam1 in NMDA Receptor-Dependent Spine/Dendrite Development

Tiam1 couples the NMDA receptor to the regulation of spine and dendrite development by inducing specific Rac1-dependent intracellular signaling pathways that control actin remodeling and mRNA translation.

(data not shown), suggesting that Tiam1 phosphorylation is complex and requires further study to determine its functional relevance. In addition to CaMKs, NMDA receptor stimulation also activates the small GTPase Ras (Yun et al., 1998). Ras activation is mediated by Ca^{2+}-regulated Ras GEFs such as Ras-GRF, a protein that interacts with the NR2B subunit of the NMDA receptor (Krapivinsky et al., 2003; Tian et al., 2004). Activated Ras may contribute to NMDA receptor-dependent Tiam1 regulation by binding to the Ras binding domain (RBD) of Tiam1 and enhancing Tiam1's GEF activity toward Rac1 (Lambert et al., 2002). Tiam1's activity may also be modulated by the Ras effector PI3K, since its lipid product, phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3), can bind to the N-terminal PH domain of Tiam1 and promote Tiam1 membrane localization (Fleming et al., 2000; Sander et al., 1998). The ability of the NMDA receptor to regulate Tiam1 activity via these pathways may be influenced by EphB receptors, which interact with NMDA receptors and potentiate NMDA receptor-dependent Ca^{2+} influx (Dalva et al., 2000; Takasu et al., 2002). Our finding that EphB activation induces the recruitment of Tiam1 to NMDA receptor complexes provides support for this possibility. EphBs may enhance Tiam1 recruitment to NMDA receptors by stimulating NMDA receptor clustering and/or by helping anchor Tiam1 at NMDA receptor complexes through a direct interaction with Tiam1 (K.F.T. and M.E.G., unpublished data).

Once activated by the NMDA receptor, Tiam1 exerts its effect on dendritic spine and branch development at least in part by stimulating the activity of its downstream target, Rac1. Rac1 is known to play a central role in the regulation of dendritic branch and spine morphology. The blockade of Tiam1 function recapitulates many of the phenotypes found with inhibition of Rac1 activity, including decreased dendritic complexity and spine density, and increased spine length (Li et al., 2000; Nakayama et al., 2000; Tashiro and Yuste, 2004). By activating Rac1 in response to NMDA receptor stimulation, Tiam1 contributes to the actin cytoskeletal remodeling that is required for dendritic arbor growth and spine and synapse development. In addition, Tiam1-induced actin remodeling may play a role in synaptic plasticity, since blocking actin dynamics with pharmacological inhibitors impairs hippocampal LTP (Fukazawa et al., 2003; Kim and Lisman, 1999). The actin cytoskeleton may contribute to the molecular mechanisms underlying LTP by regulating activity-induced spine morphogenesis (Matus, 2000), AMPA receptor trafficking (Bredt and Nicoll, 2003), and the localization and functional state of postsynaptic proteins such as neurotransmitter receptors (Gomez et al., 2002).

NMDA receptor stimulation also activates PI3K, resulting in the production of lipid second messengers that recruit and activate the protein kinases, phosphoinositide-dependent kinase 1 (PDK1) and Akt (Chan et al., 1999). However, the mechanism by which Ca^{2+} influx through NMDA receptors induces the PI3K/Akt pathway is not clear. We find that reducing Tiam1 expression with RNAi leads to an inhibition of the glutamate-induced phosphorylation/activation of Akt, indicating that Tiam1 participates in this NMDA receptor-dependent signaling pathway. The requirement for Tiam1 in NMDA receptor-dependent Akt activation is likely due to Tiam1's ability to activate Rac1, since Rac1 has been shown to act upstream of PI3K and Akt (Genot et al., 2000; Tolias et al., 1995; Weiner et al., 2002).

The finding that Tiam1 is required for NMDA receptor-dependent Akt activation is intriguing given that the PI3K/Akt pathway has been shown to play a role in the expression of hippocampal LTP (Sanna et al., 2002), fear conditioning in the amygdala (Lin et al., 2001), and NMDA receptor-dependent long-term depression (Daw et al., 2002). The PI3K/Akt pathway may affect these processes by regulating dendritic development and plasticity. This likely involves actin remodeling, since PI3K is required for the actin filament reorganization that occurs in response to a variety of extracellular stimuli (Kotani et al., 1994; Shaw et al., 1997), and Akt can modulate actin dynamics by activating the Rac effector kinase Pak (Chung et al., 2001; Tang et al., 2000). The PI3K/Akt pathway may also contribute to NMDA receptor-dependent development and plasticity by regulating protein trafficking (Carpenter and Cantley, 1996), given that PI3K is necessary for AMPA receptor insertion during LTP (Man et al., 2003).

In addition to actin remodeling and protein trafficking, the PI3K/Akt pathway also regulates mRNA translation, which is thought to play an essential role in LTP and memory (Kelleher et al., 2004; Tang et al., 2002) as well as dendritic arbor growth and spine morphogenesis (Crino and Eberwine, 1996; Ostroff et al., 2002). The PI3K/Akt pathway promotes protein synthesis by regulating proteins such as p70 S6 kinase (S6K), mTOR, and 4E-BP, which are known to control mRNA translation (Hay and Sonenberg, 2004). By triggering the phosphorylation and inactivation of 4E-BP, the PI3K/Akt pathway releases the eukaryotic initiation factor 4E (eIF4E) from the inactive eIF4E/4E-BP complex, enabling eIF4E to form an active initiation complex with

initiation factor eIF4G (Gingras et al., 2001). Thus, the finding that Tiam1 is required for the phosphorylation of 4E-BP suggests that Tiam1 plays a role in regulating NMDA receptor-dependent protein synthesis, which may be important for Tiam1's ability to modulate dendritic development and plasticity.

Through its ability to control mRNA translation, the PI3K/Akt pathway plays a critical role in the regulation of cell size (Kozma and Thomas, 2002). The requirement for Tiam1 in the NMDA receptor-dependent activation of the PI3K/Akt pathway could explain why knockdown of Tiam1 expression results in a reduction in neuronal soma size. Furthermore, by locally regulating protein synthesis at synapses, Tiam1 may contribute to the NMDA receptor-mediated modulation of dendritic spine size (Ostroff et al., 2002), a possibility that is supported by the finding that Tiam1 knockdown results in longer, more filopodia-like spines.

Neuronal activity is one of several stimuli that affect the development and plasticity of dendritic arbors and spines. Other environmental cues known to regulate dendrite and/or spine development include the neurotrophin BDNF (Huang and Reichardt, 2001), ephrins (Palmer and Klein, 2003), semaphorins (Polleux et al., 2000), Slit1 (Whitford et al., 2002), and Notch (Sestan et al., 1999). Given the key role of Rho GTPases in controlling dendritic arbor and spine development, it is possible that the effects of some of these environmental cues on dendritic development are mediated by Tiam1 activation of Rac1.

The need for precise regulation of Rho GTPase signaling within spines is highlighted by the finding that mutations in genes involved in Rho GTPase signaling are responsible for altered spine morphology in animal models (Luo et al., 1996; Meng et al., 2002) and in X-linked mental retardation in humans (Ramakers, 2002). These spine abnormalities are thought to result in defects in neuronal connectivity, thereby impairing the brain's ability to store and process information. Given the importance of Tiam1 for normal spine development, it is likely that further elucidation of mechanisms by which the activity of Tiam1 and other Rho family regulatory proteins are modulated in spines and dendrites may help to clarify the causes underlying mental retardation and other neurological disorders.

Experimental Procedures

DNA Constructs

The following regions of human Tiam1 were PCR amplified and subcloned into the ClaI/SmaI sites of the pflag-CMV2 vector (Sigma) or the SpeI/NotI sites of the pEF1-Myc-HisA vector (Invitrogen): full-length Tiam1, C1199 Tiam1 (aa 393–1590), PHn-CC-Ex (aa 393–855), PDZ (aa 840–1025), and DH-PH (aa 1025–1590). Rat Tiam1 cDNA was obtained by RT-PCR from RNA isolated from rat E18 cortical neurons (DIV7) and subcloned into the pEF1-Myc-HisA vector. Tiam1QK (Q1191A, K1195A) was generated using the QuickChange site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. The following constructs have been described previously: NR1 (Dalva et al., 2000), NR2B (Takasu et al., 2002), and Kalirin-7 (Penzes et al., 2000).

The pSUPER-Tiam1 RNAi construct was designed as described (Brummelkamp et al., 2002). Briefly, the following complementary oligonucleotides were annealed and inserted into the HindIII/BglI sites of the pSUPER vector: 5′-GATCCCCGAGGGAGAAGGAAGTG GTCTTCAAGAGAGACCACTTCCTTCTCCCTCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGAGGGAGAAGGAAGTGGTCTCTCTTGAA GACCACTTCCTTCTCCCTCTTT-3′ (targeted against bp 2212–2232 of rat Tiam1). This region was not homologous to Tiam2 or any other known genes, as determined by a BLAST search. This siRNA sequence was also cloned into the lentivirus expression vector pLenti-U6BX (Cellogenetics, Inc), and the lentivirus was generated as described (Rubinson et al., 2003). Viral titers were determined by infection of HEK 293T cells. After concentration, viral titers were 3×10^6 pfu/μl.

Antibodies

A description of the antibodies used can be found in the Supplemental Data.

Cell Cultures, Transfections, and Infections

HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (100 U/ml, 100 μg/ml, respectively). Hippocampal and cortical neurons were prepared from E18 Long-Evans rat embryos (Charles River) as described (Xia et al., 1996). For a detailed description of cell culture conditions, transfections, and infections, see the Supplemental Data.

Preparation of EphrinB1-Fc

EphrinB1-Fc (R&D Systems) and Fc were aggregated at 10 μg/ml for 45 min with 0.09 mg/ml goat anti-human Fcγ antibody (Jackson Laboratories). Neurons were then stimulated with 500 ng/ml clustered ligand for 60 min at 37°C (Dalva et al., 2000).

Immunoprecipitations and Western Blot Analysis

See the Supplemental Data for details on immunoprecipitations and Western blotting.

Immunocytochemistry and Immunohistochemistry

For details of immunocytochemistry, as well as immunohistochemistry and immunogold EM of rat brain slices, see the Supplemental Data.

Rac1 Activation Assay

Neurons (DIV7) were stimulated with 50 μM glutamate for 5 min. Rac1 activation was measured using the Rac1 activation assay kit (UBI). Briefly, extracts were incubated with 5 μl/tube of PBD resin for 1 hr at 4°C. Pellets were washed three times, and inputs and pellets were analyzed by Western blotting with anti-Rac1 antibodies.

Image Analysis and Quantification

For details of image analysis and quantification, see the Supplemental Data.

Electrophysiology

For a description of the electrophysiology, see the Supplemental Data.

Supplemental Data

The Supplemental Data include two figures and Supplemental Experimental Procedures and can be found with this article online at http://www.neuron.org/cgi/content/full/45/4/525/DC1/.

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References

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. *15*, 6541–6551.

Bonhoeffer, T., and Yuste, R. (2002). Spine motility: Phenomenology, mechanisms, and function. Neuron *35*, 1019–1027.

Bredt, D.S., and Nicoll, R.A. (2003). AMPA receptor trafficking at excitatory synapses. Neuron *40*, 361–379.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science *296*, 550–553.

Cantrell, D.A. (2001). Phosphoinositide 3-kinase signalling pathways. J. Cell Sci. *114*, 1439–1445.

Carpenter, C.L., and Cantley, L.C. (1996). Phosphoinositide kinases. Curr. Opin. Cell Biol. *8*, 153–158.

Chan, T.O., Rittenhouse, S.E., and Tsichlis, P.N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu. Rev. Biochem. *68*, 965–1014.

Chung, C.Y., Potikyan, G., and Firtel, R.A. (2001). Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. Mol. Cell *7*, 937–947.

Crino, P.B., and Eberwine, J. (1996). Molecular characterization of the dendritic growth cone: regulated mRNA transport and local protein synthesis. Neuron *17*, 1173–1187.

Cull-Candy, S., Brickley, S., and Farrant, M. (2001). NMDA receptor subunits: diversity, development and disease. Curr. Opin. Neurobiol. *11*, 327–335.

Dalva, M.B., Takasu, M.A., Lin, M.Z., Shamah, S.M., Hu, L., Gale, N.W., and Greenberg, M.E. (2000). EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell *103*, 945–956.

Daw, M.I., Bortolotto, Z.A., Saulle, E., Zaman, S., Collingridge, G.L., and Isaac, J.T. (2002). Phosphatidylinositol 3 kinase regulates synapse specificity of hippocampal long-term depression. Nat. Neurosci. *5*, 835–836.

Ehler, E., van Leeuwen, F., Collard, J.G., and Salinas, P.C. (1997). Expression of Tiam-1 in the developing brain suggests a role for the Tiam-1-Rac signaling pathway in cell migration and neurite outgrowth. Mol. Cell. Neurosci. *9*, 1–12.

Fleming, I.N., Elliott, C.M., Buchanan, F.G., Downes, C.P., and Exton, J.H. (1999). Ca2+/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation. J. Biol. Chem. *274*, 12753–12758.

Fleming, I.N., Gray, A., and Downes, C.P. (2000). Regulation of the Rac1-specific exchange factor Tiam1 involves both phosphoinositide 3-kinase-dependent and -independent components. Biochem. J. *351*, 173–182.

Fukazawa, Y., Saitoh, Y., Ozawa, F., Ohta, Y., Mizuno, K., and Inokuchi, K. (2003). Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo. Neuron *38*, 447–460.

Garcia-Gallo, M., Renart, J., and Diaz-Guerra, M. (2001). The NR1 subunit of the N-methyl-D-aspartate receptor can be efficiently expressed alone in the cell surface of mammalian cells and is required for the transport of the NR2A subunit. Biochem. J. *356*, 539–547.

Gaudilliere, B., Konishi, Y., de la Iglesia, N., Yao, G., and Bonni, A. (2004). A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. Neuron *41*, 229–241.

Genot, E.M., Arrieumerlou, C., Ku, G., Burgering, B.M., Weiss, A., and Kramer, I.M. (2000). The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositide 3-kinase. Mol. Cell. Biol. *20*, 5469–5478.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.

Goldin, M., Segal, M., and Avignone, E. (2001). Functional plasticity triggers formation and pruning of dendritic spines in cultured hippocampal networks. J. Neurosci. *21*, 186–193.

Gomez, L.L., Alam, S., Smith, K.E., Horne, E., and Dell'Acqua, M.L. (2002). Regulation of A-kinase anchoring protein 79/150-cAMP-dependent protein kinase postsynaptic targeting by NMDA receptor activation of calcineurin and remodeling of dendritic actin. J. Neurosci. *22*, 7027–7044.

Grunwald, I.C., Korte, M., Wolfer, D., Wilkinson, G.A., Unsicker, K., Lipp, H.P., Bonhoeffer, T., and Klein, R. (2001). Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. Neuron *32*, 1027–1040.

Guo, X., Lin, Y., Horbinski, C., Drahushuk, K.M., Kim, I.J., Kaplan, P.L., Lein, P., Wang, T., and Higgins, D. (2001). Dendritic growth induced by BMP-7 requires Smad1 and proteasome activity. J. Neurobiol. *48*, 120–130.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. *18*, 1926–1945.

Henderson, J.T., Georgiou, J., Jia, Z., Robertson, J., Elowe, S., Roder, J.C., and Pawson, T. (2001). The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. Neuron *32*, 1041–1056.

Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. *24*, 677–736.

Kawauchi, T., Chihama, K., Nabeshima, Y., and Hoshino, M. (2003). The in vivo roles of STEF/Tiam1, Rac1 and JNK in cortical neuronal migration. EMBO J. *22*, 4190–4201.

Kelleher, R.J., 3rd, Govindarajan, A., and Tonegawa, S. (2004). Translational regulatory mechanisms in persistent forms of synaptic plasticity. Neuron *44*, 59–73.

Kim, C.H., and Lisman, J.E. (1999). A role of actin filament in synaptic transmission and long-term potentiation. J. Neurosci. *19*, 4314–4324.

Kotani, K., Yonezawa, K., Hara, K., Ueda, H., Kitamura, Y., Sakaue, H., Ando, A., Chavanieu, A., Calas, B., Grigorescu, F., et al. (1994). Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. EMBO J. *13*, 2313–2321.

Kozma, S.C., and Thomas, G. (2002). Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K. Bioessays *24*, 65–71.

Krapivinsky, G., Krapivinsky, L., Manasian, Y., Ivanov, A., Tyzio, R., Pellegrino, C., Ben-Ari, Y., Clapham, D.E., and Medina, I. (2003). The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. Neuron *40*, 775–784.

Lambert, J.M., Lambert, Q.T., Reuther, G.W., Malliri, A., Siderovski, D.P., Sondek, J., Collard, J.G., and Der, C.J. (2002). Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. Nat. Cell Biol. *4*, 621–625.

Li, Z., Van Aelst, L., and Cline, H.T. (2000). Rho GTPases regulate distinct aspects of dendritic arbor growth in Xenopus central neurons in vivo. Nat. Neurosci. *3*, 217–225.

Li, Z., Aizenman, C.D., and Cline, H.T. (2002). Regulation of rho GTPases by crosstalk and neuronal activity in vivo. Neuron *33*, 741–750.

Lin, C.H., Yeh, S.H., Lu, K.T., Leu, T.H., Chang, W.C., and Gean, P.W. (2001). A role for the PI-3 kinase signaling pathway in fear conditioning and synaptic plasticity in the amygdala. Neuron *31*, 841–851.

Lin, H., Huganir, R., and Liao, D. (2004). Temporal dynamics of NMDA receptor-induced changes in spine morphology and AMPA

receptor recruitment to spines. Biochem. Biophys. Res. Commun. *316*, 501–511.

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. *3*, 175–190.

Luo, L. (2002). Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. Annu. Rev. Cell Dev. Biol. *18*, 601–635.

Luo, L., Hensch, T.K., Ackerman, L., Barbel, S., Jan, L.Y., and Jan, Y.N. (1996). Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature *379*, 837–840.

Man, H.Y., Wang, Q., Lu, W.Y., Ju, W., Ahmadian, G., Liu, L., D'Souza, S., Wong, T.P., Taghibiglou, C., Lu, J., et al. (2003). Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. Neuron *38*, 611–624.

Matus, A. (2000). Actin-based plasticity in dendritic spines. Science *290*, 754–758.

Meng, Y., Zhang, Y., Tregoubov, V., Janus, C., Cruz, L., Jackson, M., Lu, W.Y., MacDonald, J.F., Wang, J.Y., Falls, D.L., and Jia, Z. (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron *35*, 121–133.

Merlot, S., and Firtel, R.A. (2003). Leading the way: Directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. J. Cell Sci. *116*, 3471–3478.

Michiels, F., Stam, J.C., Hordijk, P.L., van der Kammen, R.A., Ruuls-Van Stalle, L., Feltkamp, C.A., and Collard, J.G. (1997). Regulated membrane localization of Tiam1, mediated by the NH2-terminal pleckstrin homology domain, is required for Rac-dependent membrane ruffling and C-Jun NH2-terminal kinase activation. J. Cell Biol. *137*, 387–398.

Moon, S.Y., and Zheng, Y. (2003). Rho GTPase-activating proteins in cell regulation. Trends Cell Biol. *13*, 13–22.

Nakayama, A.Y., Harms, M.B., and Luo, L. (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. J. Neurosci. *20*, 5329–5338.

Nikonenko, I., Jourdain, P., Alberi, S., Toni, N., and Muller, D. (2002). Activity-induced changes of spine morphology. Hippocampus *12*, 585–591.

Ostroff, L.E., Fiala, J.C., Allwardt, B., and Harris, K.M. (2002). Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. Neuron *35*, 535–545.

Palmer, A., and Klein, R. (2003). Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. Genes Dev. *17*, 1429–1450.

Penzes, P., Johnson, R.C., Alam, M.R., Kambampati, V., Mains, R.E., and Eipper, B.A. (2000). An isoform of kalirin, a brain-specific GDP/GTP exchange factor, is enriched in the postsynaptic density fraction. J. Biol. Chem. *275*, 6395–6403.

Penzes, P., Johnson, R.C., Sattler, R., Zhang, X., Huganir, R.L., Kambampati, V., Mains, R.E., and Eipper, B.A. (2001). The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. Neuron *29*, 229–242.

Perez-Otano, I., Schulteis, C.T., Contractor, A., Lipton, S.A., Trimmer, J.S., Sucher, N.J., and Heinemann, S.F. (2001). Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. J. Neurosci. *21*, 1228–1237.

Perkinton, M.S., Ip, J.K., Wood, G.L., Crossthwaite, A.J., and Williams, R.J. (2002). Phosphatidylinositol 3-kinase is a central mediator of NMDA receptor signalling to MAP kinase (Erk1/2), Akt/PKB and CREB in striatal neurones. J. Neurochem. *80*, 239–254.

Polleux, F., Morrow, T., and Ghosh, A. (2000). Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature *404*, 567–573.

Ramakers, G.J. (2002). Rho proteins, mental retardation and the cellular basis of cognition. Trends Neurosci. *25*, 191–199.

Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Zhang, M., McManus, M.T., Gertler, F.B., Scott, M.L., and Van Parijs, L. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. *33*, 401–406.

Sander, E.E., van Delft, S., ten Klooster, J.P., Reid, T., van der Kammen, R.A., Michiels, F., and Collard, J.G. (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J. Cell Biol. *143*, 1385–1398.

Sanna, P.P., Cammalleri, M., Berton, F., Simpson, C., Lutjens, R., Bloom, F.E., and Francesconi, W. (2002). Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. J. Neurosci. *22*, 3359–3365.

Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. *16*, 1587–1609.

Sestan, N., Artavanis-Tsakonas, S., and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science *286*, 741–746.

Shaw, L.M., Rabinovitz, I., Wang, H.H., Toker, A., and Mercurio, A.M. (1997). Activation of phosphoinositide 3-OH kinase by the $\alpha 6\beta 4$ integrin promotes carcinoma invasion. Cell *91*, 949–960.

Sholl, D.A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. J. Anat. *87*, 387–406.

Sin, W.C., Haas, K., Ruthazer, E.S., and Cline, H.T. (2002). Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. Nature *419*, 475–480.

Sone, M., Hoshino, M., Suzuki, E., Kuroda, S., Kaibuchi, K., Nakagoshi, H., Saigo, K., Nabeshima, Y., and Hama, C. (1997). Still life, a protein in synaptic terminals of Drosophila homologous to GDP-GTP exchangers. Science *275*, 543–547.

Sone, M., Suzuki, E., Hoshino, M., Hou, D., Kuromi, H., Fukata, M., Kuroda, S., Kaibuchi, K., Nabeshima, Y., and Hama, C. (2000). Synaptic development is controlled in the periactive zones of Drosophila synapses. Development *127*, 4157–4168.

Stam, J.C., Sander, E.E., Michiels, F., van Leeuwen, F.N., Kain, H.E., van der Kammen, R.A., and Collard, J.G. (1997). Targeting of Tiam1 to the plasma membrane requires the cooperative function of the N-terminal pleckstrin homology domain and an adjacent protein interaction domain. J. Biol. Chem. *272*, 28447–28454.

Steward, O., and Schuman, E.M. (2001). Protein synthesis at synaptic sites on dendrites. Annu. Rev. Neurosci. *24*, 299–325.

Steward, O., and Schuman, E.M. (2003). Compartmentalized synthesis and degradation of proteins in neurons. Neuron *40*, 347–359.

Takasu, M.A., Dalva, M.B., Zigmond, R.E., and Greenberg, M.E. (2002). Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. Science *295*, 491–495.

Tang, Y., Zhou, H., Chen, A., Pittman, R.N., and Field, J. (2000). The Akt proto-oncogene links Ras to Pak and cell survival signals. J. Biol. Chem. *275*, 9106–9109.

Tang, S.J., Reis, G., Kang, H., Gingras, A.C., Sonenberg, N., and Schuman, E.M. (2002). A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proc. Natl. Acad. Sci. USA *99*, 467–472.

Tashiro, A., and Yuste, R. (2004). Regulation of dendritic spine motility and stability by Rac1 and Rho kinase: evidence for two forms of spine motility. Mol. Cell. Neurosci. *26*, 429–440.

Tian, X., Gotoh, T., Tsuji, K., Lo, E.H., Huang, S., and Feig, L.A. (2004). Developmentally regulated role for Ras-GRFs in coupling NMDA glutamate receptors to Ras, Erk and CREB. EMBO J. *23*, 1567–1575.

Tolias, K.F., Cantley, L.C., and Carpenter, C.L. (1995). Rho family GTPases bind to phosphoinositide kinases. J. Biol. Chem. *270*, 17656–17659.

Van Aelst, L., and Cline, H.T. (2004). Rho GTPases and activity-dependent dendrite development. Curr. Opin. Neurobiol. *14*, 297–304.

Weiner, O.D., Neilsen, P.O., Prestwich, G.D., Kirschner, M.W., Cantley, L.C., and Bourne, H.R. (2002). A PtdInsP(3)- and Rho GTPase-

mediated positive feedback loop regulates neutrophil polarity. Nat. Cell Biol. *4*, 509–513.

West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., and Greenberg, M.E. (2001). Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA *98*, 11024–11031.

West, A.E., Griffith, E.C., and Greenberg, M.E. (2002). Regulation of transcription factors by neuronal activity. Nat. Rev. Neurosci. *3*, 921–931.

Whitford, K.L., Marillat, V., Stein, E., Goodman, C.S., Tessier-Lavigne, M., Chedotal, A., and Ghosh, A. (2002). Regulation of cortical dendrite development by Slit-Robo interactions. Neuron *33*, 47–61.

Wong, R.O., and Ghosh, A. (2002). Activity-dependent regulation of dendritic growth and patterning. Nat. Rev. Neurosci. *3*, 803–812.

Xia, Z., Dudek, H., Miranti, C.K., and Greenberg, M.E. (1996). Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. *16*, 5425–5436.

Yun, H.Y., Gonzalez-Zulueta, M., Dawson, V.L., and Dawson, T.M. (1998). Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. Proc. Natl. Acad. Sci. USA *95*, 5773–5778.