Synaptic Plasticity, a Symphony in GEF

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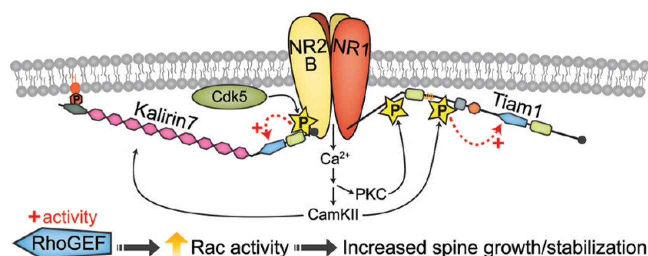
Abstract



Dendritic spines are the postsynaptic sites for the majority of excitatory synapses in the mammalian forebrain. While many spines display great stability, others change shape in a matter of seconds to minutes. These rapid alterations in dendritic spine number and size require tight control of the actin cytoskeleton, the main structural component of dendritic spines. The ability of neurons to alter spine number and size is essential for the expression of neuronal plasticity. Within spines, guanine nucleotide exchange factors (GEFs) act as critical regulators of the actin cytoskeleton by controlling the activity of Rho-GTPases. In this review, we focus on the Rho-GEFs expressed in the nucleus accumbens and localized to the postsynaptic density and, thus, positioned to effect rapid alterations in the structure of dendritic spines. We review literature that ties these GEFs to different receptor systems and intracellular signaling cascades and discuss the effects these interactions are likely to have on synaptic plasticity.

Keywords: Actin filament; dendritic spine; post-synaptic density; RhoGTPase; Rac1; RhoA

In the mammalian forebrain, the vast majority of excitatory synapses (75−90%) terminate on dendritic spines (*1*, *2*). While many of these spines are stable, lasting for months even *in vivo*, one of the key hallmarks of spines is their lability. Dendritic spine size changes in response to a variety of extracellular stimuli. Of particular importance, stimuli that induce long-term potentiation, believed to be the cellular correlate of learning, result in increases in both spine size and spine density in *ex vivo* slice preparations (*3*−*7*). Additionally, animals that have undergone training in a fear conditioning paradigm have altered dendritic spine content and morphology in the amygdala, the brain region responsible for this type of learning (*8*, *9*).

While alterations in dendritic spine plasticity are important for learning and memory, it is likely that they play a role in pathological plasticity as well. Chronic treatment with cocaine or amphetamine increases spine density in the nucleus accumbens (NAc), while chronic morphine treatment decreases spine density in this crucial region (*10*−*14*). In addition to addiction, various forms of mental retardation and psychiatric conditions such as schizophrenia have been linked to changes in spine morphology (*15*−*20*). For example, patients with fragile X syndrome demonstrate a profound increase in spine density (*21*−*24*), while a number of nonsyndromic mental retardations show decreases in spine density (*25*, *26*). Down's syndrome patients have significantly decreased dendritic spine density, branching, and length in various cortical subregions (*15*, *27*). Clearly, tight regulation of spine formation is crucial for normal neuronal function.

Key to the regulation of dendritic spine formation and function is the actin cytoskeleton, which makes up the structural core of the spine (*6*, *28*−*32*). Actin dynamics are crucial to the plasticity involved in forming and reshaping new spines. Rho-GTPases, a subfamily of the small GTP-binding proteins, are critical for regulation of the actin cytoskeleton and myriad other cellular functions (*6*, *32*−*36*). We focus here on the signaling pathways used to control these molecular switches at the postsynaptic density.

Control of the Actin Cytoskeleton: Rho Proteins

Rho-GTPases function as tightly regulated molecular switches. In their basal state, these proteins are GDP-bound and inactive. Activation is controlled by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. GTP-bound Rho-GTPases are active and can form high-affinity interactions with their downstream targets, regulating target protein activity and localization (*37*). GTPase activating proteins (GAPs), which accelerate the GTPase

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activity of their target Rho proteins, return the Rho proteins to their inactive, GDP-bound state. While there are 61 Rho-GEFs and 68 Rho-GAPs encoded by the human genome, there are only 20 Rho-family GTPases (*38*) (http://www.uniprot.org/). The gene tallies differ slightly in the mouse, with 58 Rho-GEFs, 56 Rho-GAPs, and 20 Rho-GTPases (http://www.uniprot.org/). In both species, the excess of GEFs and GAPs over GTPases presents an interesting regulatory quagmire and puts a premium on sites of expression and subcellular localization.

Since we are interested in understanding how drugs of abuse hijack the mechanisms that control synaptic plasticity, we focus on the nucleus accumbens (NAc), a region of the ventral striatum containing primarily GABAergic medium spiny neurons that receive heavy dopaminergic innervation from the ventral tegmental area and glutamatergic inputs from the prefrontal cortex (*39*, *40*). Based on extensive behavioral data, the nucleus accumbens is frequently called the "reward center" of the brain (*41*−*46*). mRNA-Seq technology (*47*) was used to identify 31 Rho-GEF, 26 Rho-GAP, and 14 Rho-GTPase genes whose transcripts are expressed at significant levels in the adult mouse NAc (Eipper-Mains, J. E., unpublished observations). Official gene names, synonyms, and associated identifiers for all annotated Rho-GEF, Rho-GAP and Rho-GTPase genes in the mouse genome are provided in Supplementary Table 1, Supporting Information.

The epicenter of the dendritic spine is the postsynaptic density (PSD), an electron dense structure composed of over 500 proteins, including scaffolds, ligand-gated ion channels, receptors, and signaling molecules (*48*−*50*). In order to focus on the Rho-GEFs, GAPs, and GTPases that are localized to the PSD, we compared our list of expressed transcripts to the forebrain PSD proteome (*51*−*53*). Ten of the Rho-GEFs expressed at significant levels in the NAc have been identified in purified PSDs or otherwise localized to the PSD (*54*) (J. E.-M., unpublished observations). Official gene names, synonyms, and SMART structures of the major neuronal isoforms for these Rho-GEFs are shown in Figure 1; five terminate with a PDZ-binding motif and five include an SH3 domain. Of the Rho-GTPases expressed in the NAc, only Rac1 and RhoG have been localized to the PSD (J. E.-M., unpublished observations). Localization to the PSD puts these Rho-GEF/Rho-GTPase pairs in a unique position to respond rapidly to extracellular stimuli and modify the actin cytoskeleton in a manner dependent on synaptic activity. Of the Rho-GAPs expressed in the NAc, only three are known to localize to the PSD (AU040829, Bcr, D15Wsu169e) (J. E.-M., unpublished observations). While Rho-GAPs clearly play an integral part in the regulation of Rho-GTPase activity, their role in synaptic plasticity is outside the scope of this review. We focus our review on how the PSD-localized Rho-GEFs are regulated by extracellular and intracellular signals and the roles they might play in modulating dendritic spine function.

With respect to dendritic spine dynamics, the best-characterized Rho GTPases are Rac1, RhoA, and Cdc42. Within spines, Rac1 and RhoA seem to play antagonistic roles, with Rac1 promoting spine formation and growth and RhoA promoting spine retraction and decreases in spine density (*33*, *55*, *56*). Cdc42 also promotes spine formation and is present in spines (*19*, *57*) but has not been localized to the PSD. We begin with a review of the Rho-GEFs that preferentially activate Rac1 before moving on to those that activate RhoA and Cdc42.

Rac1-Specific Rho-GEFS

Tiam1

Tiam1 (T-cell lymphoma invasion and metastasis 1) is a Rac1-specific GEF with a single Dbl homology−pleckstrin homology (DH-PH) domain near its C-terminus, a PDZ-binding motif at its C-terminus, and an additional PH domain closer to its N-terminus (Figure 1) (*58*). Additionally, Tiam1 has an internal PDZ domain that binds to the C-terminal peptide sequence Tyr-Tyr-(Phe/Ala), although neuronal interaction partners have not yet been identified (*58*). When expressed in fibroblasts, Tiam1 induces membrane ruffling and a morphology resembling that of constitutively activated Rac1 (*59*). Interestingly, it is the more N-terminal PH domain of Tiam1 that is essential for its membrane localization, and deletion of this domain leads to cytosolic dispersal and a lack of induced membrane changes (*60*). Studies in neuroblastoma lines demonstrated that overexpression of Tiam1 increases Rac1-dependent neurite outgrowth and insensitivity to RhoA-induced neurite retraction (*61*). While Tiam1 was not identified in purified rat forebrain PSDs (*51*, *52*), immunogold electron microscopy demonstrates that Tiam1 is intercalated into the PSD (*54*). This study, which was the first to examine Tiam1 in primary neurons, demonstrated that Tiam1 is necessary for the elaboration of a full dendritic arbor. Neurons transfected with RNAi targeted against Tiam1 showed a dramatic reduction in spine density, and the remaining spines were longer and more filopodial than spines in control cultures (*54*).

Neurons expressing Tiam1 RNAi also exhibited a sharp decrease in miniature postsynaptic currents, indicating a functional as well as a morphological change (*54*). The ability of Tiam1 to interact directly with the NR1 NMDA receptor subunit, but not with any of the NR2 subunits, presumably contributes to this deficit (Figure 2a). Stimulation of NMDA receptors in control

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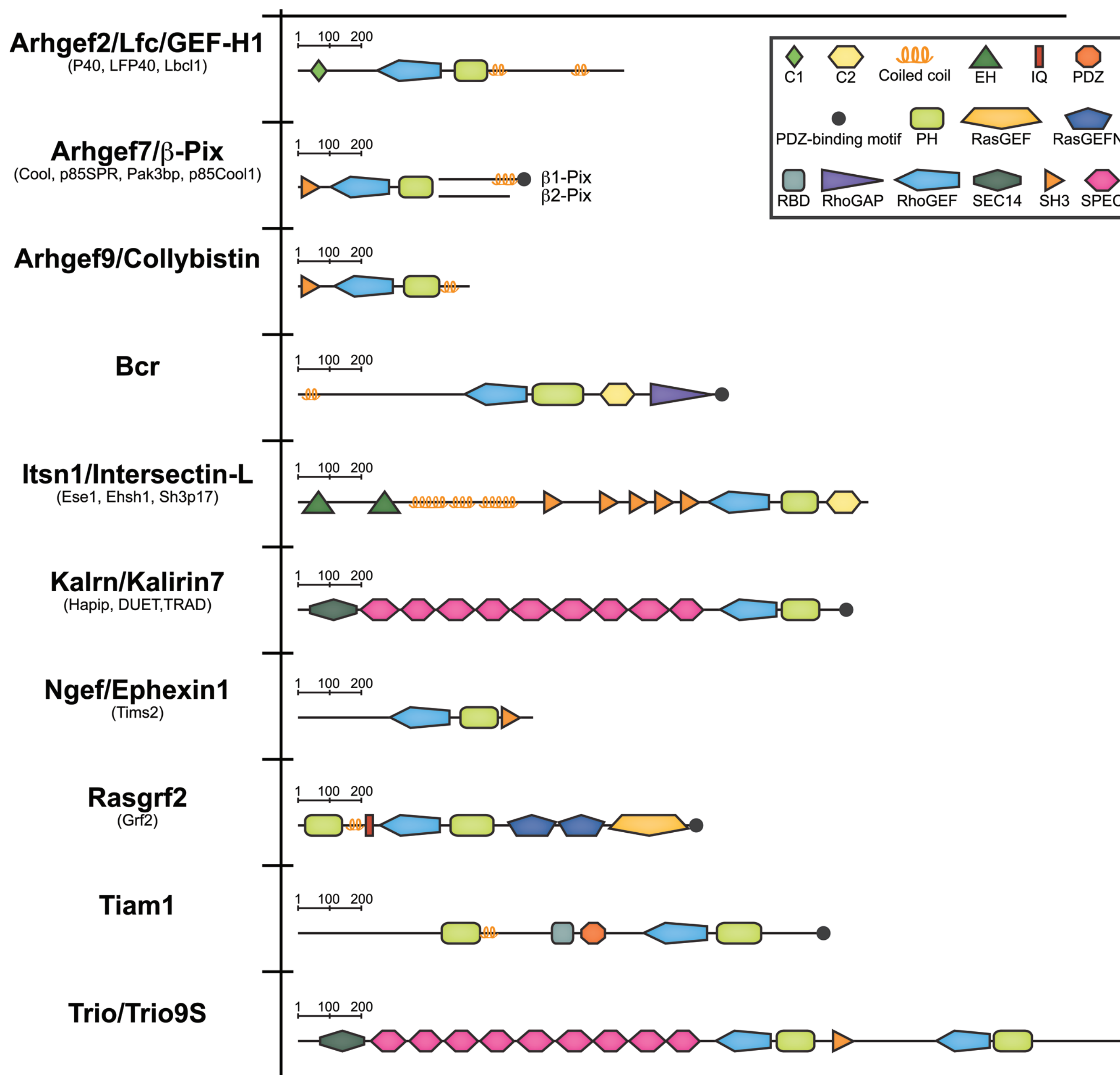

Figure 1. Rho-GEFs and Rho-GTPases expressed in the NAc and localized to the PSD. Gene diagrams are drawn to scale and represent SMART structures (http://smart.embl-heidelberg.de/) with several modifications (see Supplementary Table 2).

cultures leads to an increase in spine density and size; neurons expressing Tiam1 RNAi showed no such changes in spine morphology (*54*). This result is particularly compelling because it presents a strong functional connection for the interaction of Tiam1 and NR1. Further linking NMDA receptors to Tiam1 signaling, Tiam1 is phosphorylated on unknown threonine residues by CamKII and protein kinase C (PKC), enhancing its GEF activity about 2-fold (*62*, *63*) (Figure 2a). Given that CamKII and PKC are indirectly activated by calcium (*64*, *65*) and both represent major components of the PSD (*51*), the anchoring of Tiam1 to NMDA receptors may enhance the response of the synapse to the calcium influx that results from NMDA receptor stimulation (*66*).

In addition to NMDA receptors, Tiam1 also forms a stable interaction with the receptor tyrosine kinase EphB2 (*67*) (Figure 2a). EphB2 is stimulated by its presynaptic binding partner EphrinB, increasing dendritic spine number and size (*68*). Stimulation of cultured

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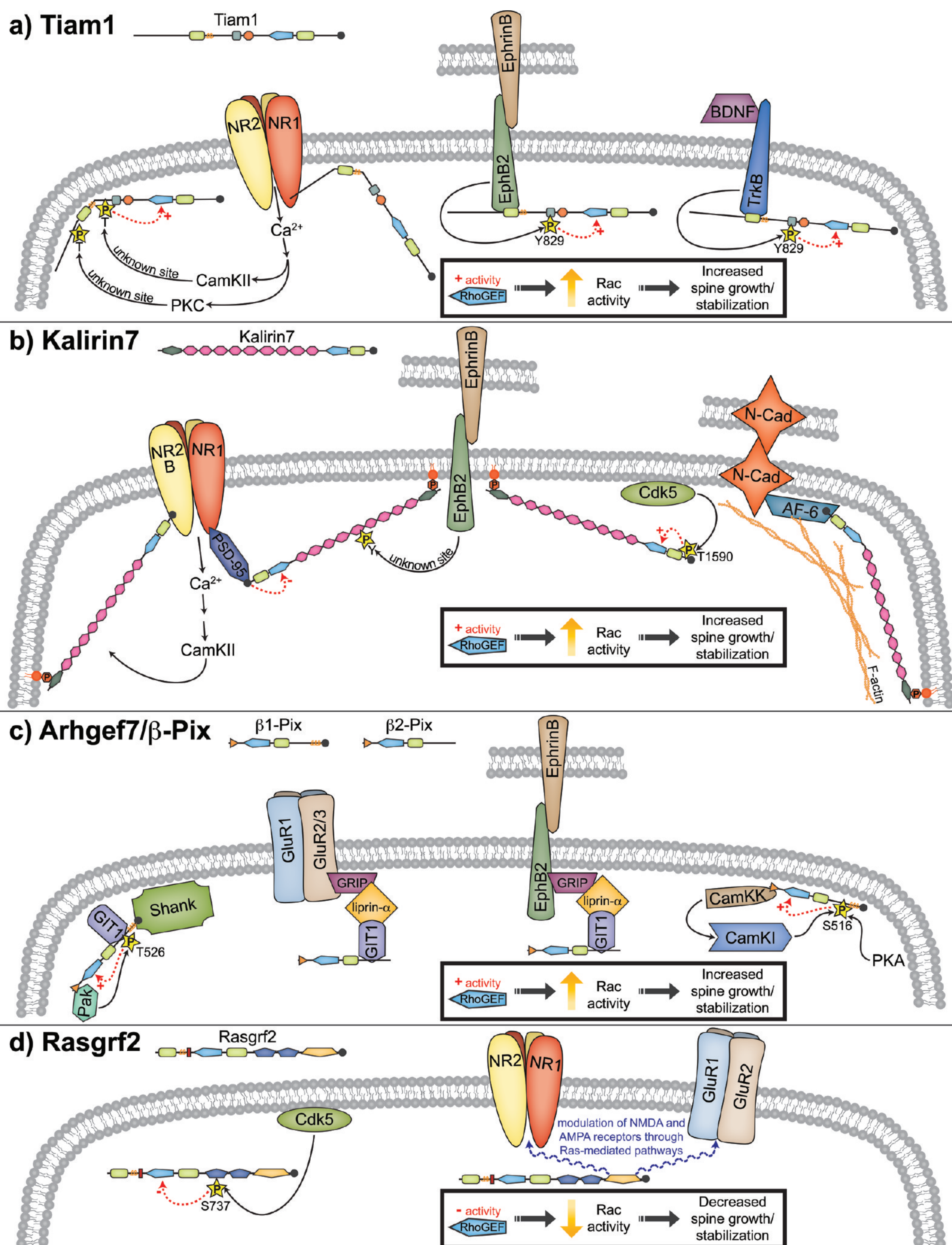

Figure 2. Rac-specific GEFs at the PSD: (a) Tiam1 with NR1 subunit of NMDA receptor, EphB, and TrkB; (b) Kalirin7 with PSD-95, PI-3-P/PI-3,5-P2, NR2B subunit of NMDA receptor, EphB, Cdk5, and AF-6/N-cadherin; (c) Arhgef7/β-Pix with CamKK/CamKI/PKA, GluR2/3 subunit of AMPA receptor via GIT1/liprin-α/GRIP, Pak, Shank, and EphB; (d) Rasgrf2 with Cdk5, NMDA, and AMPA receptors.

neurons with EphrinB leads to a clustering of Tiam1 at the sites of new synaptic contacts. At these sites, Tiam1 and EphB2 form a stable complex with EphB2 binding to the N-terminal PH domain of Tiam1. As a result,

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EphB2 phosphorylates Tiam1 at Tyr[829] (*67*) (Figure 2a). In COS7 cells, a phosphomimetic point mutant at this site leads to an increase in Rac1 activation (*69*). Importantly, RNAi knockdown of Tiam1 ablates the ability of activated EphB2 to increase spine formation in cultured hippocampal neurons, indicating that this pathway is Tiam1-dependent (*67*). The interaction of Tiam1 with both NMDA receptors and EphB2 begins to paint an interesting picture of an important molecular complex at the PSD. EphB2 has repeatedly been shown to be important for NMDA receptor clustering and function (*70*−*73*). Additionally, stimulation of both EphB2 (*68*, *70*) and NMDA receptors (*74*) has been implicated in the growth and formation of new spines. As a direct interactor with both proteins, Tiam1 is well-positioned to integrate voltage-sensitive, ligand-gated ion channel signaling and receptor tyrosine kinase signaling to effect activity-dependent changes in the actin cytoskeleton.

Finally, Tiam1 interacts with another receptor tyrosine kinase, TrkB (*69*) (Figure 2a). The binding of BDNF to TrkB has long been known to be important for the formation and maturation of synapses (*75*). Tiam1 interacts with TrkB via its N-terminal PH domain and is phosphorylated on Tyr[829] (as with EphB2) (*69*). Expression of a non-phosphorylatable Tiam1 mutant (Tyr[829]Phe) blocked the BDNF-induced increase in dendritic spines, demonstrating an essential role for phosphorylation at this site (*69*). Although a Tiam1 knockout mouse has been created and is viable (*76*), it has primarily been used for cancer research.

Kalirin7

In the adult brain, Kalirin7, with a single GEF domain specific for Rac1 and RhoG, is the most abundant splice variant of the *Kalrn* gene (*77*, *78*) (Figure 1); several larger Kalirin isoforms, with a second GEF domain specific for RhoA, are expressed at lower levels but are not localized to the PSD (*79*−*82*). Kalirin7 terminates with a PDZ-binding motif, and it is estimated that the average hippocampal PSD, which contains about 20 NMDA receptors and 15 AMPA receptors (*48*), contains 10 copies of Kalirin7 (*83*). At the N-terminus of Kalirin7 is a Sec14p domain that binds phosphatidylinositol-3-phosphate and phosphatidylinositol-3,5-bisphosphate and is essential for membrane localization (*84*); this is followed by nine spectrin-like repeats (Figures 1 and 2b).

A yeast two-hybrid screen with the PDZ-binding motif of Kalirin7 identified 16 PDZ domain-containing interactors (*85*). Given that these PDZ domain-containing proteins interact with a variety of receptor subtypes (e.g., NMDA, AMPA, 5-HT), protein kinases (e.g., PKA, CamKII), and filamentous actin, this places Kalirin7 in a unique position to integrate a wide variety of signals at the PSD (*86*). The interaction of Kalirin7 with PSD-95 decreases its ability to activate Rac1 (Figure 2b). Kalirin7 was identified in the NMDA receptor interactome after affinity purification using a peptide from the C-terminus of the NR2B subunit (*51*); this interaction was verified by coimmunoprecipitation of Kalirin7 with NR2B (Kiraly, D. D., unpublished observations). Consistent with this, PSDs purified from mice lacking Kalirin7 have decreased levels of NR2B (*83*). Unlike Tiam1, Kalirin7 does not coimmunoprecipitate with NR1 (*54*). Tiam1, through its association with the NR1 subunit (*54*), and Kalirin7, through its association the NR2B subunit, may serve to modulate critical signaling steps downstream of specific NMDA receptor subsets.

The ability of Kalirin7 to increase spine formation *in vitro* has been demonstrated in cultured cortical and hippocampal neurons (*85*, *87*, *88*). Perhaps most compelling, Kalirin7 expression in otherwise aspiny interneurons leads to the formation of spine-like structures that acquire apposing presynaptic terminals and appear to function as active synapses (*89*). Recently, these findings were validated *in vivo* with the development of a Kalirin7 knockout mouse. These mice display a decrease in dendritic spine density in the hippocampus as well as impaired hippocampal LTP and focal hippocampus-dependent learning impairments (*83*). Much has been done to clarify the intracellular molecular interactors with Kalirin7. In cultured cortical neurons, Kalirin7 is essential for EphB2-induced spine formation (*88*, *90*) (Figure 2b). Stimulation of cell cultures with clustered EphrinB led to a modest tyrosine phosphorylation signal on Kalirin7, although the location of the phosphorylated residue was not established (*88*). Kalirin7 and Tiam1 may work in parallel pathways to propagate signals downstream of this receptor tyrosine kinase.

It has been suggested that the N-terminus (Thr[95]) of Kalirin7 is phosphorylated by CamKII and that phosphorylation at this site is essential for Kalirin7 to display GEF activity (*91*). However, mutation of Thr[95], which is not a consensus CamKII site (*64*, *92*, *93*), to a non-phosphorylatable alanine (Thr[95]Ala) did not eliminate its GEF activity, and the GEF activity of Kalirin7 and shorter splice variants lacking this region (ΔKal7) are indistinguishable *in vitro* (*84*, *94*, *95*). Coimmunoprecipitation of Kalirin7 and GluR1 led Xie and colleagues to hypothesize that Kalirin7 is necessary for the movement of GluR1 into newly formed dendritic spines upon NMDA receptor stimulation of cortical cultures (*91*). Mice genetically lacking Kalirin7 have normal levels of GluR1 in both tissue homogenates and purified PSDs (*83*), so Kalirin7 is not essential for AMPA receptor localization *in vivo*.

The Kalirin7 PDZ-binding motif interacts with the PDZ domains of AF-6/Afadin, Neurabin, and Spinophilin, each

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of which can bind directly to filamentous actin. AF-6 also mediates the interaction of Kalirin7 with N-cadherin, a trans-synaptic protein that forms homophilic interactions with presynaptic N-cadherin (*85*, *96*) (Figure 2b). N-cadherin plays an important role in the regulation of dendritic spine morphology (*97*, *98*). Stimulation of neuronal cultures with clustered N-cadherin results in an increase in dendritic spine size and recruits AF-6, Kalirin7, and Rac1 into dendritic spines (*96*). When expression of all isoforms of Kalirin was reduced using RNAi, clustered N-cadherin was no longer able to change dendritic spine morphology (*96*).

Kalirin7 is phosphorylated at a single site (Thr^{1590}) by Cdk5 (Figure 2b), a synaptically localized serine/threonine kinase known to play an important role in synapse development and modulation; Cdk5 is localized to membranes and activated by binding to p35 or p39 but not to a cyclin (*95*, *99*−*106*). Phosphorylation of Thr^{1590} increases the GEF activity of Kalirin, with the phosphomimetic mutant (Thr^{1590}Asp) showing a 2-fold increase in activity over the non-phosphorylatable mutant (Thr^{1590}Ala). Mutation of Thr^{1590} to Asp or Ala did not affect the ability of Kalirin7 to increase spine density. While neurons expressing Thr^{1590}Asp-Kalirin7 exhibited increased dendritic spine size, neurons expressing Thr^{1590}Ala-Kalirin7 did not (*95*). The fact that Kalirin7 knockout mice show a decrease in Cdk5 levels in purified synaptosomes and PSDs (*83*) suggests a role for reciprocal regulation of Kalirin7 and Cdk5 *in vivo* as well as *in vitro*.

Trio

Trio, a paralog of Kalirin, plays essential roles both within and outside of the nervous system but is not localized to the PSD. Trio is a critical player in axon guidance and nervous system development (*107*−*112*). Mice genetically lacking Trio do not survive much beyond birth; their inability to form a Trio/M-cadherin/Rac1 complex may contribute to the observed skeletal muscle deficits (*113*, *114*). Deficits were also apparent in hippocampal and olfactory bulb organization in $Trio^{-/-}$ mice. The fact that $Trio^{-/-}$ cortical neurons do not respond normally to netrin-1, failing to exhibit the expected axonal extension via Rac1-mediated actin cytoskeleton growth (*107*), may contribute to these deficits. Its first GEF domain is specific for Rac1/RhoG and its second for RhoA (Figure 1). The DH domain of p63Rho-GEF (GEFT) closely resembles the second DH domain of Trio and Kalirin, and all three enzymes exhibit basal inhibition through their associated PH domains. Structural and genetic studies revealed a major role for activated $G_{\alpha q}$ in relieving this autoinhibition by binding to the extended PH domain (*115*, *116*).

Arhgef7/*β*-Pix

Arhgef7 (*β*-Pix) was identified based on its ability to activate Rac1, Cdc42, and Pak (p21 activated kinase) (*117*) (Figure 2c). Two isoforms of *β*-Pix, *β*1 and *β*2, which differ only at their C-termini, are the major ones expressed in the mammalian brain (*118*). The *β*1 isoform C-terminus has a coiled-coil domain and a PDZ-binding motif, while the *β*2 isoform C-terminus has a serine-rich region (Figure 2c) (*118*). The N-terminal SH3 domain of *β*-Pix binds to Pak, recruiting Pak to Rac1- and Cdc42-induced adhesion complexes; mutations that inactivate this SH3 domain lead to mislocalization of Pak (*117*). Crystal structure analysis detailed the interactions of the SH3 domain of *β*-Pix with Pak (*119*). *β*-Pix is phosphorylated by Pak on Thr^{526}, increasing its membrane localization and boosting its GEF activity, ultimately eliciting neurite formation in PC12 cells (*120*).

When studied in primary hippocampal cultures, it became clear that *β*-Pix is recruited to synapses by GIT1 (G-protein-coupled receptor-kinase-interacting protein 1), which is present in the PSD (*51*, *121*). GIT1 is a ubiquitously expressed ARF-GAP (ADP-ribosylation factor GTPase activating protein) that is enriched at the PSD and complexes with AMPA receptor subunits in the rat brain through its association with liprin-α (*122*). Liprin-α is a multifunctional protein with a number of known interacting proteins including the GRIP/ABP family (glutamate receptor interacting proteins) of multi-PDZ domain containing proteins. Through their PDZ domains, GRIPs at the PSD interact with a number of different proteins including the AMPA receptor subunits GluR2 and GluR3, the receptor tyrosine kinase EphB2 and the neuronal Ras-GEF GRASP-1 (*122*, *123*).

The GIT1-binding domain of *β*-Pix lies just upstream of the C-terminal coiled-coil and the C-terminal serine-rich regions of *β*1- and *β*2-Pix, respectively (*118*). The ability of GIT1 to recruit *β*-Pix to synapses is necessary for the formation of new dendritic spines with apposing presynaptic terminals. When the *β*-Pix-binding domain of GIT1 was deleted, *β*-Pix showed a more diffuse localization, leading to a multitude of aberrant cell protrusions that were not associated with presynaptic terminals. The GEF activity of *β*-Pix is necessary for the formation of new spine-like structures in hippocampal neurons (*121*). GIT1 is not solely responsible for the localization of *β*-Pix to synapses. *β*1-Pix binds to the PDZ domain of Shank via its C-terminal PDZ-binding motif (*49*) or its coiled-coil domain (*124*), and overexpression of Shank recruits additional *β*-Pix to dendritic spines (*124*) (Figure 2c), blocking actin bundling (*125*). While *β*-Pix does not bind directly to the PDZ domains of PSD95, Sap97, S-Scam, GRIP1, Lin7, or Densilin, it directly interacts with the PDZ domain of Scribble, a protein most studied in cancer biology and

epithelial polarity; in PC12 cells stimulated with KCl, Scribble recruits β-Pix to the plasma membrane (*126*).

β-Pix modulates Ca^{2+}-dependent intracellular signaling. β-Pix coimmunoprecipitates with CamKK (CamK-kinase) in transfected cells and rat brain lysates (*127*). This interaction is mediated via the SH3 domain of β-Pix, the region also known to interact with Pak. β-Pix is phosphorylated by CamKI, a downstream target of CamKK, on Ser^{516}, a site known to be phosphorylated by PKA (*128*). The GEF activity of β-Pix is stimulated in a Ca^{2+}-dependent manner, and mutation of Ser^{516} to a non-phosphorylatable mutant (Ser^{516}Ala) obviated the Ca^{2+}-dependent changes in Rac1 activation (*127*). Expression of dominant negative CaMKI, Ser^{516}Ala-βPIX, or siRNA for either CaMKI or β-Pix resulted in a marked decrease in dendritic spine density in cultured neurons (*127*). This study clearly demonstrates a role for β-Pix in calcium−calmodulin signaling cascades.

While β-Pix has not been directly linked to any cell-surface receptor in mammals, deletion of the corresponding *Drosophila* gene leads to decreased synaptic levels of the glutamate receptor subunit GluRIIA (*129*). In mice, β-Pix is linked to GluR2/3 AMPA receptor subunits via the GIT1/liprin-α/GRIP complex described above. Disruption of the GIT1/liprin-α interaction results in decreased AMPA receptor clustering in cultured neurons (*122*). Taken together, the *Drosophila* and mouse data suggest a role for β-Pix in proper postsynaptic AMPA receptor targeting via its interaction with GIT1/liprin-α/GRIP. Additionally, GIT1 is indirectly recruited to synapses by EphrinB stimulation of the EphB2 receptor tyrosine kinase (*130*). Three PSD Rho-GEFs, β-Pix, Tiam1, and Kalirin7, mediate morphogenic signals downstream of EphB2 (*67*, *88*, *131*, *132*).

Rasgrf2

Rasgrf2 is unique in that it contains both a Rho-GEF DH−PH domain, as well as a Ras-GEF domain (Figure 1). Rasgrf2 has a C-terminal type II PDZ-binding motif with no known binding partners (*38*). Ras promotes the creation of larger spines receiving excitatory synapses and is normally held in check by SynGAP (*133*). Interestingly, Rasgrf2 has been linked to the Cdk5/p35 signaling pathway in neurons. Cdk5 phosphorylates Rasgrf2 on Ser^{737}, decreasing its Rac1-GEF activity, but has no effect on its ability to activate Ras (*134*) (Figure 2d). Additionally, while Rasgrf2 is typically found along dendrites in basal conditions, coexpression of p35 and Cdk5 leads to the retraction of Rasgrf2 into the cell soma (*134*). This is another indication of the many ways in which Cdk5 activity affects PSD-GEF function. The Ras-GEF domain of Rasgrf2 has been implicated in the control of both NMDA and AMPA glutamate receptors but will not be discussed here as we are focusing on Rho-GEFs at the PSD (*135*−*137*).

RhoA/Cdc42-Specific Rho-GEFs

Ngef/Ephexin1

In the NAc, transcripts encoding Ngef (Ephexin1) are more prevalent than those encoding any other Rho-GEF (J. E.-M., unpublished observations). Ephexin1 has C-terminally located DH, PH, and SH3 domains (Figure 1). Ephexin1 activates Rac1, Cdc42, and RhoA (*138*); different signaling pathways shift its specificity toward Rac1/Cdc42 or toward RhoA, altering the downstream response. Ephexin1-mediated activation of Rac1 has been studied in the context of axon outgrowth and neuronal development (*138*−*141*). While the events leading to Rac1 activation by the Rho-GEFs discussed above are generally well characterized, the events involved in Ephexin1-mediated Rac1 and Cdc42 activation are not well understood. Pak, which is involved in β-Pix activation of Rac1, demonstrates increased activity in the context of Ephexin1-mediated axonal outgrowth (*138*). Pak may complex with Ephexin1 and shift its GEF activity toward Rac1/Cdc42 activation and actin polymerization (*138*) (Figure 2a).

A great deal more is known about the role of Ephexin1 in RhoA activation. The generation of an Ephexin1 knockout mouse facilitated studies into the role of Ephexin1 in axonal outgrowth and growth cone collapse in cultured retinal ganglion cells (RGCs) (*139*). $Ephexin^{-/-}$ RGCs exhibit the same number of neurites per cell but have significantly shorter axons than their wild-type counterparts (*139*). Incubation of $Ephexin1^{-/-}$ RGCs with EphrinA1 does not elicit the wild-type phenotype of growth cone collapse, implicating an EphA4/Ephexin1 interaction in the control of EphrinA1-induced growth cone collapse. It should not go unmentioned that Ephexin1 knockout mice show no overt axonal phenotype *in vivo* (*139*), perhaps due to functional redundancy among Rho-GEFs.

The DH−PH domain of Ephexin1 binds the kinase domain of EphA4 in cultured fibroblasts (*138*); a direct interaction between EphA4 and Ephexin1 has not been demonstrated by coimmunoprecipitation in neurons (*139*). Ephexin1 is phosphorylated following EphrinA1 stimulation of EphA4 receptors, and this phosphorylation event is required for growth cone collapse in cultured RGCs (*139*). Stimulation of the EphA4 receptor by EphrinA1 in neuronal cultures results in EphA4 autophosphorylation and recruitment of two kinases, Cdk5/p35 and Src, to EphA4 at the PSD (*104*, *140*). EphrinA1 binding causes a loss of dendritic spines and a corresponding decrease in miniature excitatory postsynaptic current (mEPSC) frequency (*104*). The EphA4 receptor coimmunoprecipitates with Cdk5/p35

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from rat brain extracts, suggesting a direct interaction *in vivo* (*104*).

Activated EphA4 phosphorylates the recruited Cdk5, stimulating its kinase activity and its subsequent phosphorylation of Ephexin1 at three sites, Thr^{41}, Thr^{47}, and Ser^{139} (*104*) (Figure 3a). Mutant Ephexin1, in which the Cdk5 target residues are replaced with non-phosphorylatable alanine residues, eliminates the ability of Src to phosphorylate Ephexin1 on Tyr^{87}; Ephexin1 with glutamate at these same sites supports robust EphrinA1/EphA4-mediated Ephexin1 phosphorylation at Tyr^{87} (*104*). The kinase domain of EphA4 does not directly phosphorylate Ephexin1 at Tyr^{87} (*139*). Rather, EphA4 and Src demonstrate ligand-induced association with subsequent stimulation of the tyrosine kinase activity of Src (*140*). Activated Src phosphorylates Ephexin1 at Tyr^{87}, an event that shifts the GEF activity of Ephexin1 toward RhoA and ultimately results in growth cone collapse (*139*, *142*) (Figure 3a). Inhibition of Src-dependent phosphorylation of Ephexin1 at Tyr^{87} obviates EphrinA1/EphA4-induced repulsive axon guidance in retinal axons (*140*). Similarly, analysis of hippocampal cultures from $Cdk5^{-/-}$ mice revealed an obligatory role for Cdk5 in the EphrinA1-mediated loss of dendritic spines and decrease in mEPSC frequency (*104*).

There is evidence in cultured cells that the FGF and EphA receptors form hetero-oligomers and cross-activate one another (*143*). Activated FGF receptor phosphorylates Ephexin1 at several tyrosine residues, including Tyr^{87}, and shifts its GEF activity toward RhoA (*142*). While the FGF receptor is known to be present at presynaptic terminals and is expressed in astrocytes (*144*), it is not present at the PSD (*51*, *52*) and is thus unlikely to play a major role in dendritic spine actin dynamics.

Arhgef2/Lfc/GEF-H1

Arhgef2 (Lfc; GEF-H1) is a RhoA-specific GEF with a single DH−PH domain and two coiled coil regions near its C-terminus (*145*) (Figure 1). Under basal conditions Lfc is found predominantly in dendritic shafts, where it interacts with microtubules via its PH domain (*145*, *146*). In cultured neurons, NMDA receptor activation causes the rapid translocation of Lfc into dendritic spines (*146*) (Figure 3b). A recent study examining Lfc localization in primate cortex at the electron micrograph level also concluded that Lfc was primarily localized to dendritic shafts under basal conditions, translocating rapidly into spines upon synaptic stimulation (*147*). Once in spines, Lfc interacts with Spinophilin and Neurabin, which bind filamentous actin (*146*) (Figure 3b). One study, by Ryan and colleagues, demonstrated that overexpression of Lfc in cultured neurons produces a decrease in spine size and an increase in spine density (*146*). Consistent with this, a more recent study by Kang and colleagues demonstrated that expression of dominant negative Lfc or shRNA targeted to Lfc increased spine size (*148*). However, in the more mature cultures studied by Kang and colleagues, reductions in Lfc action increased spine density, perhaps suggesting developmental alterations the Lfc/RhoA pathway (*148*). Activation of RhoA has typically been associated with decreased spine density (*33*, *149*).

Even under basal conditions, Kang and colleagues found Lfc in the PSD, a finding supported by PSD proteomics studies (*51*, *52*). Based on both proteomic analyses of AMPA receptor complexes and coimmunoprecipitation with the GluR1 and GluR2 AMPA receptor subunits, Lfc interacts with AMPA receptors. Incubation of hippocampal cultures with an AMPA receptor antagonist normally leads to a decrease in dendritic spine density; in neurons expressing Lfc-shRNA, this effect is ablated (*148*). Suppression of AMPA receptor function normally increases RhoA activity; this response is diminished when Lfc expression is reduced using Lfc-shRNA, and stimulation of AMPA-R inhibits Lfc activity and RhoA signaling (*148*) (Figure 3b). Lfc seems to play a crucial role in RhoA-mediated spine retraction.

The GEF activity of Lfc is also controlled by phosphorylation (*150*−*152*). Phosphorylation by kinases Aurora A/B (at Ser^{885}) or Cdk1/Cyclin B (at Ser^{959}) decreases Lfc-Rho-GEF activity (*150*), while phosphorylation by ERK1/2 (at Thr^{678}) enhances Lfc-Rho-GEF activity (*151*). PKA phosphorylates Ser^{885}, with concomitant AKAP121 binding to Lfc in HEK293 cells; the phospho-specific scaffolding protein, 14-3-3, then binds to P-Ser^{885}-Lfc, decreasing the GEF activity of Lfc (*153*) (Figure 3b). Though proteomics studies have localized PKA to the PSD (*51*, *52*), the role of PKA-dependent phosphorylation in the regulation of Lfc-Rho-GEF activity in neurons remains to be studied.

Arhgef9/Collybistin

Arhgef9 (Collybistin) is a brain-specific Rho-GEF with a single DH−PH domain that is specific for Cdc42 (*154*) (Figure 1). Two isoforms of Collybistin are expressed in brain: Collybistin1 includes an N-terminal SH3 domain and a C-terminal coiled-coil domain; Collybistin2 lacks these two domains (*155*). Although the identities of the specific players regulating Collybistin-Rho-GEF activity and localization remain elusive, it is clear that its SH3 domain plays a key role in these interactions (*156*).

Collybistin is expressed broadly throughout the forebrain and cerebellum from early embryonic life through adulthood (*155*, *157*). Collybistin was originally discovered as an interactor with Gephyrin, a protein essential for the formation of inhibitory postsynaptic structures in brain and spinal cord (*154*, *155*). The linker region between the SH3 and DH domains is essential for the

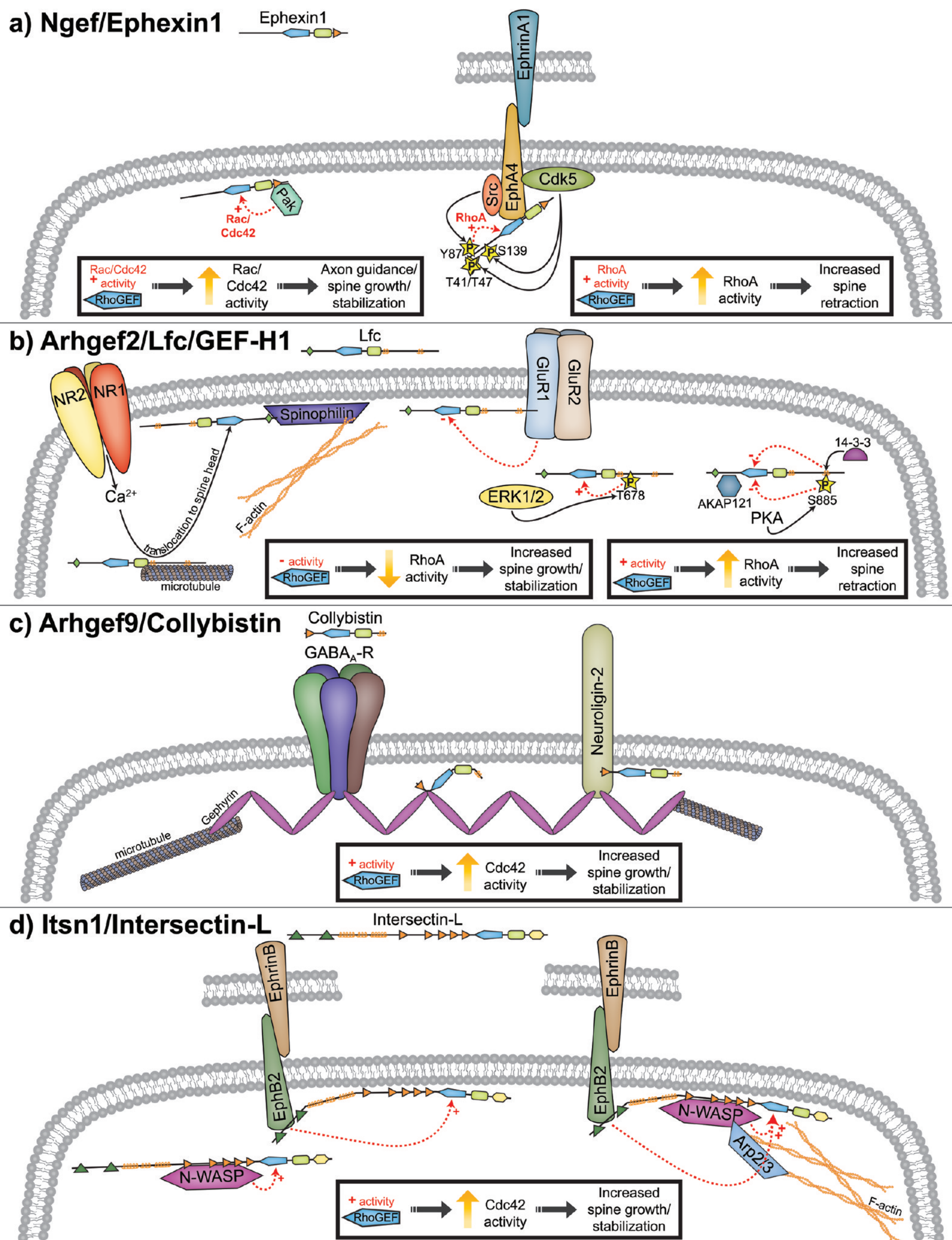

Figure 3. Rho/Cdc42-specific GEFs at the PSD: (a) Ngef/Ephexin1 with Pak, EphA4, Src, and Cdk5; (b) Arhgef2/Lfc/GEF-H1 with a microtubule, Spinophilin, GluR1 subunit of AMPA receptor, ERK1/2, AKAP121/PKA, and 14-3-3; (c) Arhgef9/Collybisitin with Gephyrin and Neuroligin-2; (d) Itsn1/Intersectin-L with N-WASP, EphB2, and Arp2/3.

Collybistin−Gephyrin interaction (*158*) (Figure 3c). Collybistin with an active PH domain and an intact Gephyrin-binding site is essential for the postsynaptic localization of Gephyrin in dendrites of cultured cortical

neurons (*156*). A patient exhibiting hyperekplexia and epilepsy, both potentially caused by a decrease in inhibitory neurotransmission, had a mutation that disrupted the SH3 region of Collybistin (*156*). Expression of this mutant protein in cultured cortical neurons led to a loss of $GABA_A$ receptor clustering (*156*). Another patient with a balanced chromosomal translocation leading to disruption of the Collybistin gene displayed disturbed sleep cycles, increased anxiety, epileptic seizures, and mental retardation (*159*). Collybistin clearly plays a key role in the regulation of inhibitory transmission in humans as well as rodents.

The Collybistin knockout mouse displays normal locomotor activity, but anxiety-like behavior is increased dramatically, and spatial learning is grossly impaired (*160*). In the hippocampus and amygdala of the Collybistin$^{-/-}$ mouse, $GABA_A$ receptor expression is greatly reduced and inhibitory neurotransmission is decreased. Interestingly, these mice display enhanced CA1 LTP in hippocampal slices, while LTD plasticity is impaired (*160*). In contrast, induction of LTP in the dentate gyrus of Collybistin$^{-/-}$ mice is greatly decreased (*161*). Both Collybistin and Gephyrin interact with Neuroligin-2, a trans-synaptic scaffolding protein essential for the formation of inhibitory synapses (*162*) (Figure 3c). The interaction of Collybistin and Neuroligin-2 enhances the ability of Collybistin to recruit Gephyrin to synapses, which in turn leads to the formation of more inhibitory synapses in cultured neurons (*162*). Taken together, these studies demonstrate a clear role for Collybistin in the normal formation of inhibitory synapses and the balance of excitatory/inhibitory transmission in the mammalian forebrain but leave unanswered the role of the GEF domain of Collybistin in the nervous system.

Itsn1/Intersectin-L

The longer neuron-specific splice variant of the *Itsn1* gene, Intersectin-L, includes C-terminal DH, PH, and C2 domains as well as several common protein−protein interacting regions it shares with the shorter, ubiquitously expressed splice variant, Intersectin-S (*163*−*165*) (Figure 1). The common region includes two Eps15 homology domains, several coiled-coil regions, and five SH3 domains. Intersectin-S affects neuronal endocytic vesicle formation (*165*). The tandem DH−PH domains of Intersectin-L interact specifically with Cdc42 to stimulate its GTPase activity, and overexpression of Intersectin-L induces formation of filopodia in fibroblasts (*163*).

Regulation of the Rho-GEF activity of Intersectin-L occurs at many levels. Activity assays demonstrate more robust GEF activity in C-terminal DH−PH tail constructs than in full-length Intersectin-L, suggesting a role for N-terminal modulation of the GEF activity of Intersectin-L. N-WASP (neuronal Wiskott−Aldrich syndrome protein), which activates the Arp2/3 actin-nucleating complex, interacts directly with the SH3 domains of Intersectin-L, stimulating its GEF activity; the Intersectin-L/N-WASP complex can be immunoprecipitated from transfected cells and from native brain extracts (*163*) (Figure 3d). The N-WASP/Intersectin-L interaction relieves Intersectin-L autoinhibition and may stabilize the GEF domain interaction with GDP-bound Cdc42, catalyzing its activation (*163*). Additionally, the kinase domain of the receptor tyrosine kinase EphB2 interacts with the N-terminus of Intersectin-L, activating Cdc42 in hippocampal neurons; the Intersectin-L/EphB2 complex can be immunoprecipitated from extracts of transfected cells (*166*) (Figure 3d).

N-WASP links Cdc42 to the actin cytoskeleton via its ability to stimulate the Arp2/3 complex, causing actin nucleation (*31*, *163*, *167*). Colocalization of EphB2 and N-WASP with Intersectin-L and F-actin in dendritic spines suggests a model in which EphB2 and N-WASP act synergistically to activate Intersectin-L GEF activity (*165*, *166*) (Figure 3d). Concomitant binding of N-WASP to the Intersectin-L/Cdc42 complex results in relief of N-WASP autoinhibition and a dose-dependent N-WASP-mediated stimulation of Intersectin-L GEF activity (*31*, *163*). Activated N-WASP stimulates the Arp2/3 complex, causing actin polymerization (*31*, *163*). N-WASP plays roles both up- and downstream of Intersectin-L, highlighting the importance that multimolecular complexes play in the regulation of the actin cytoskeleton (*168*).

The *Itsn1* gene has been implicated in Down syndrome (DS), with DS neurons exhibiting abnormal dendritic spine morphology and altered endosomal pathways (*164*). The *Itsn1* gene is located on human chromosome 21, the same chromosome present in extra copy number in Down syndrome, further suggesting a role for *Itsn1* in the pathophysiology of Down syndrome (*165*).

Bcr

Bcr (Breakpoint cluster region) negatively regulates cell proliferaton and oncogenic transformation (*169*). Bcr and the closely related protein Abr are the only two GTPase-activating proteins that specifically inactivate Rac1 (*170*). Bcr both turns Rac1 on (via its GEF domain) and inactivates Rac1 (via its Rho-GAP domain) (Figure 1). Bcr exhibits GEF activity toward Rac1, Cdc42, and RhoA (*38*). In addition to its DH−PH and GAP domains, Bcr has a serine/threonine kinase domain and a C-terminal PDZ-binding motif that interacts with the PDZ domain of AF-6/Afadin, a protein known to bind Ras and filamentous actin (*169*). The kinase domain of Bcr phosphorylates AF-6 near its PDZ domain, promoting a direct interaction between

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Bcr and AF-6 (*169*). While the impact of the Bcr/AF-6 interaction on the GEF activity of Bcr remains to be determined, the existence of this phosphorylation-mediated interaction raises the possibility that phosphorylation events can regulate interactions between other PDZ-binding motif-containing Rho-GEFs and their PDZ-domain-containing partners (*38*).

Bcr is among a set of genes expressed very early in development (*171*). Full-length Bcr contributes to NGF-independent neurite formation in PC12 cells (*172*), while a fusion construct including the GEF domain but not the Rho-GAP domain suppresses neurite outgrowth (*172*). Mice in which both Bcr and Abr are knocked out show a number of glial cell developmental abnormalities, which then precipitate neuronal abnormalities in the cerebellum and midbrain (*173*). A genomic translocation resulting in a fusion between Bcr and the Abl kinase is seen in 95% of patients with chronic myelogenous leukemia (CML) (*174*).

Conclusions

As primary regulators of the actin cytoskeleton, Rho-GEFs play the role of conductors in the elegant symphony that is synaptic plasticity. A preponderance of human and animal studies demonstrate that a dynamic balance of synapse formation and elimination is necessary for normal cognition and neuronal function. The delicate balance that is struck between GEF-GTPase activity and the actin cytoskeleton includes several GEFs regulated by the same pathways. A number of GEFs have been shown to be downstream of NMDA receptors (Tiam1, Kalirin7, Rasgrf2, Lfc) or Ephrin receptors (Tiam1, Kalirin7, β-Pix, Ephexin1, Intersectin-L), two receptor systems critically linked to the plasticity of the postsynaptic neuron (*132*, *175*). Additionally, while all of these proteins have been linked to a variety of intracellular signaling cascades, it is noteworthy that a number of them (Kalirin7, Rasgrf2, Ephexin1) have been linked to Cdk5, a protein known to have myriad effects on the structure and function of PSDs and spines (*100*, *104*, *106*, *176*). Additionally, Tiam1, Kalirin7, and β-Pix have all been linked to calcium−calmodulin kinase signaling pathways, which have a plethora of functions at the synapse and play essential roles in synaptic plasticity (*64*, *127*). Clearly, this puts a premium on localization of the GEF, its target GTPase, and any other signaling molecules that influence their function.

One of the quandaries raised by this review of the literature is the disparity between cell culture studies and *in vivo* models of these Rho-GEFs. While cell culture studies may indicate that a specific Rho-GEF is "essential" for dendritic spine formation (*54*, *87*), the phenotype observed in the corresponding knockout animal is often much less profound. Taking Kalirin7 as an example, cultures expressing shRNA for Kalirin7 have almost no normal dendritic spines (*89*); however, Kalirin7 knockout mice display only a 20% decrease in hippocampal spine density. While neither the cell culture nor the constitutive knockout system is without its drawbacks, the disparity between these types of studies suggests that serious consideration should be made before interpreting any of these findings in a translational or clinical sense. As new and developing technologies (mRNA-Seq, proteomics, phosphoproteomics, etc.) become more affordable and accessible to researchers, they will facilitate the integration of seemingly solitary signaling pathways into the overlapping web of pathways that is likely occurring *in vivo*.

While extensive research has pushed our understanding of dendritic spines forward, there is clearly still much more that needs to be determined. Localizing activated Rho-GEFs and Rho-GTPases and understanding the kinetics of their activation and inactivation will be critical. It will be important to clarify the conditions under which different receptors activate different Rho-GEFs and how they interact with their target Rho-GTPases. It is only through the clarification of these networks that we will be able to determine their precise role in mental retardation, drug addiction, schizophrenia, or the host of other human pathologies that demonstrate altered synaptic connectivity.

Abbreviations

C1, protein kinase C conserved region 1 (C1); C2, protein kinase C conserved region 2 (CalB); EH, Eps15 homology domain; IQ, short calmodulin-binding motif containing conserved isoleucine and glutamine residues; PDZ, domain present in PSD-95, Dlg, and ZO-1/2; PH, pleckstrin homology domain; Ras-GEF, guanine nucleotide exchange factor for Ras-like small GTPases; Ras-GEFN, guanine nucleotide exchange factor for Ras-like GTPases, N-terminal motif; RBD, Raf-like Ras-binding domain; Rho-GAP, GTPase-activator protein for Rho-like GTPases; Rho-GEF, guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases; SEC14, domain in homologues of a *Saccharomyces cerevisiae* phosphatidylinositol transfer protein (Sec14p); SH3, Src homology 3 domains; SPEC, spectrin-like repeats.

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Supporting Information Available

Tables providing names and accession numbers for all identified M. musculus Rho- GEFS, Rho-GAPs, and Rho-GTPases expressed in the PSD and modifications to SMART structures of Rho-GEFs in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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