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Control of synapse development and plasticity by Rho GTPase regulatory proteins

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Abstract

Synapses are specialized cell-cell contacts that mediate communication between neurons. Most excitatory synapses in the brain are housed on dendritic spines, small actin-rich protrusions extending from dendrites. During development and in response to environmental stimuli, spines undergo marked changes in shape and number thought to underlie processes like learning and memory. Improper spine development, in contrast, likely impedes information processing in the brain, since spine abnormalities are associated with numerous brain disorders. Elucidating the mechanisms that regulate the formation and plasticity of spines and their resident synapses is therefore crucial to our understanding of cognition and disease. Rho-family GTPases, key regulators of the actin cytoskeleton, play essential roles in orchestrating the development and remodeling of spines and synapses. Precise spatio-temporal regulation of Rho GTPase activity is critical for their function, since aberrant Rho GTPase signaling can cause spine and synapse defects as well as cognitive impairments. Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) and inhibited by GTPase-activating proteins (GAPs). We propose that Rho-family GEFs and GAPs provide the spatiotemporal regulation and signaling specificity necessary for proper Rho GTPase function based on the following features they possess: (i) existence of multiple GEFs and GAPs per Rho GTPase, (ii) developmentally regulated expression, (iii) discrete localization, (iv) ability to bind to and organize specific signaling networks, and (v) tightly regulated activity, perhaps involving GEF/GAP interactions. Recent studies describe several Rho-family GEFs and GAPs that uniquely contribute to spinogenesis and synaptogenesis. Here, we highlight several of these proteins and discuss how they occupy distinct biochemical niches critical for synaptic development.

1. Introduction: the formation and remodeling of excitatory synapses

The human brain is composed of approximately 100 billion neurons that process and transmit information in the form of electric signals. Communication between neurons occurs at specialized sites of contact called synapses. The majority of excitatory synapses on principal neurons in the brain are located on the tips of dendritic spines, small protrusions on the surface of dendrites (Fig. 1). In recent years, it has become clear that spines are dynamic

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structures that undergo rapid remodeling important for synapse formation, function and plasticity (Bhatt *et al.*, 2009; Bourne and Harris, 2008). During early postnatal development, dendritic protrusions first appear as long, thin, highly motile filopodia, which can initiate synaptic contacts with nearby axons (Dailey and Smith, 1996; Fiala *et al.*, 1998; Papa *et al.*, 1995; Ziv and Smith, 1996). As development proceeds, dendritic filopodia are replaced by (or mature into) more stable mushroom-shaped spines, which are either maintained into adulthood or eliminated (Engert and Bonhoeffer, 1999; Fiala *et al.*, 1998; Lippman and Dunaevsky, 2005; Ziv and Smith, 1996). Following development, spines continue to remodel in response to a variety of physiological stimuli (Engert and Bonhoeffer, 1999; Lendvai *et al.*, 2000; Maletic-Savatic *et al.*, 1999; Toni *et al.*, 1999). For example, synaptic activity that induces long-term potentiation (LTP), a long-lasting enhancement of synaptic strength, promotes spine enlargement and new spine formation (Matsuzaki *et al.*, 2004), whereas activity that induces long-term depression (LTD), a persistent weakening of synaptic strength, causes spine shrinkage or retraction (Zhou *et al.*, 2004). This synaptic remodeling is thought to be important for neural circuit plasticity associated with learning and memory (Yuste and Majewska, 2001). Since spine structure and synaptic function are intimately related (Kasai *et al.*, 2003), it is easy to imagine that improper spine morphogenesis might result in impaired information processing in the brain. Indeed, spine abnormalities are associated with numerous neurodevelopmental, neuropsychiatric, and neurodegenerative disorders (Fiala *et al.*, 2002; Newey *et al.*, 2005). It is therefore essential to understand the mechanisms that control the development and remodeling of spiny synapses under normal and pathological conditions.

Spines are highly enriched in filamentous actin (F-actin), and their ability to change shape depends on the rapid remodeling of the spine actin cytoskeleton (Cingolani and Goda, 2008; Honkura *et al.*, 2008). It is therefore not surprising that Rho GTPases, a subfamily of small GTP-binding proteins known for their ability to control actin cytoskeletal dyanamics, have emerged as key regulators of spine morphogenesis (Fig. 2) (Govek *et al.*, 2005). The best-studied Rho GTPase family members are RhoA, Rac1 and Cdc42. In neurons, Rac1 and Cdc42 promote the formation, growth and maintenance of spines, whereas RhoA induces spine retraction and loss (Newey *et al.*, 2005). Interestingly, mutations in a number of genes involved in Rho GTPase signaling have been linked to non-syndromic mental retardation, an intellectual disability (ID) associated with spine anomalies (Newey *et al.*, 2005; Ramakers, 2002). This apparent correlation between altered Rho GTPase signaling, spine abnormalities, and mental retardation suggests that precise Rho GTPase signaling is important for proper circuit development and normal cognitive function.

Rho GTPases regulate spine morphogenesis and synapse development by functioning as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state (Fig. 2). In their active conformation, Rho GTPases interact with specific effector molecules, which induce downstream signaling pathways that control a diverse array of biological processes including actin cytoskeletal reorganization, microtubule dynamics, gene transcription, and membrane trafficking (Govek *et al.*, 2005). Precise spatio-temporal control of Rho GTPase signaling is orchestrated by guanine nucleotide exchange factors (GEFs), which activate Rho GTPases by catalyzing GDP/GTP exchange (Schmidt and Hall, 2002) and GTPase-activating proteins (GAPs), which inhibit Rho GTPases by enhancing their intrinsic GTPase activities (Bernards and Settleman, 2004). Guanine nucleotide dissociation inhibitors (GDIs) also regulate Rho GTPases by preventing GDP/GTP exchange and sequestering inactive Rho GTPases in the cytoplasm (DerMardirossian and Bokoch, 2005). In addition to controlling Rho GTPase activity, GEFs and GAPs contribute to Rho GTPase signaling specificity by interacting with particular upstream receptors and downstream effectors (Buchsbaum *et al.*, 2002, 2003; Jaffe *et al.*, 2005; Tolias *et al.*, 2005; Tolias *et al.*, 2007; Zhang and Macara, 2008). Recent studies have identified a number of

Rho-family GEFs and GAPs that play important roles in spine morphogenesis and synapse development (Kiraly *et al.*, 2010). Determining how these GEFs and GAPs are regulated and the distinct functions they serve at synapses will be essential for developing a more mechanistic understanding of synapse formation and remodeling.

2. Regulation of Rho GTPase signaling pathways at synapses

Rho GTPases regulate a variety of neurodevelopmental processes including neuronal migration, axon growth and guidance, dendritic arborization and synaptogenesis (Govek *et al.*, 2005; Linseman and Loucks, 2008). Rho GTPases control these diverse processes by functioning at distinct locations within neurons at different developmental stages and in response to a variety of extracellular signals. The ability of Rho GTPases to act at different times and places in response to different stimuli and generate distinct cellular outcomes suggests a level of specificity that cannot reside in the GTPases alone. If, however, signaling specificity was provided by Rho GTPase regulatory proteins, we would expect these proteins to have some or all of the following features: (i) more than one GEF and/or GAP should exist for each Rho GTPase, (ii) their expression should be developmentally regulated, (iii) they should have discrete localizations within cells, (iv) they should have the capacity to organize specific Rho GTPase signaling networks, i.e. by binding to effectors and/or other signaling molecules, and (v) GEFs and GAPs should have tightly regulated activity and potentially interact with each other, at least indirectly. These features would allow a single Rho GTPase to specifically regulate multiple signaling pathways in the same cell and at the same time in a way that changes with development and is tightly regulated spatially and temporally. Do Rho GTPase GEFs and GAPs possess these features?

A general overview of Rho-family regulatory proteins indicates that they do possess characteristics that would enable them to provide specificity to Rho GTPase signaling (Moon and Zheng, 2003; Rossman *et al.*, 2005; Schmidt and Hall, 2002). First, Rho-family GEFs and GAPs both outnumber Rho GTPases by at least three- to four-fold. Second, their expression levels appear to be developmentally regulated. Third, GEFs and GAPs display varied subcellular localization profiles. Fourth, GEFs and GAPs are typically large proteins that possess multiple signaling domains that enable them to receive diverse upstream inputs as well as recruit downstream components of Rho GTPase-regulated pathways—they are both signal integrators and scaffolds (Fig. 3). Finally, the activities of Rho GTPase GEFs and GAPs are tightly regulated and emerging evidence suggests that they work closely together to control Rho GTPase activation. All of these features suggest that Rho GTPase regulatory proteins are capable of spatially and temporally regulating specific GTPase-mediated signaling pathways. Thus, while at first glance it seems that different Rho-family GEFs or GAPs have identical roles, their chief similarity is their ability to activate or inhibit specific Rho GTPases. The context of that activation or deactivation—the signal(s) to which it responds, the downstream mechanisms, and ultimate outcomes, exhibit striking diversity.

In this review, we will highlight a number of Rho-family GEFs and GAPs that are involved in synaptogenesis. We will discuss how these Rho GTPase regulatory proteins contribute to the spatiotemporal regulation and signaling specificity of Rho GTPases by examining their localization, developmental regulation, and network of protein interactions. In this way, we will argue that each Rho GTPase regulatory protein serves a unique role in the formation of synapses in the central nervous system (CNS). In order to disucuss their unique function at synapses, we have focused on better characterized GEFs and GAPs. Reports of additional Rho-family regulatory proteins involved in synaptogenesis exist, however, adequate information is not currently available in the literature for us to delineate a specific role for them at this time.

3. Rho-family GEFs involved in excitatory synaptogenesis

3.1. Rac/Cdc42-GEFs at excitatory synapses

3.1.1 Kalirin-7—The Rac-GEF Kalirin-7 has emerged as a key regulator of spine dynamics (Penzes and Jones, 2008). Kalirin-7 is produced by the *KALRN* gene, which generates several Kalirin isoforms via alternative splicing (Johnson *et al.*, 2000). Kalirin-7 is the most abundant splice variant of the *KALRN* gene in the adult brain, and its expression during development correlates with synaptogenesis (Johnson *et al.*, 2000; Penzes *et al.*, 2000). Like most Rho family GEFs, Kalirin-7 possesses multiple domains, including a lipid-binding Sec14p domain, nine spectrin-like repeats, a Rac-GEF domain consisting of a tandem Dbl homology (DH)-pleckstrin homology (PH) domain, and a C-terminal PDZ binding motif (Fig. 3). Kalirin-7 localizes to dendritic spines and is enriched in the postsynaptic density (PSD), where it interacts with numerous PDZ domain-containing proteins, including PSD-95, SAP-102, and SAP-97 (Penzes *et al.*, 2000; Penzes *et al.*, 2001). These adapter proteins link Kalirin-7 with a variety of receptor subtypes and signaling molecules at the PSD, placing Kalirin-7 in a position to integrate diverse signals that regulate different aspects of spine morphogenesis (Penzes and Jones, 2008).

The ability of Kalirin-7 to promote spine formation has been demonstrated in cultured cortical and hippocampal pyramidal neurons as well as in inhibitory aspiny interneurons, where overexpression of Kalirin-7 induces the formation of spiny synapses (Ma *et al.*, 2003; Ma *et al.*, 2008; Penzes *et al.*, 2003; Penzes *et al.*, 2001). Conversely, reduction of Kalirin-7 expression in cultured neurons by antisense RNA or RNA interference (RNAi) results in decreased spine density and loss of excitatory synapses (Ma *et al.*, 2003; Xie *et al.*, 2007). *In vivo* support for the role of Kalirin-7 in synapse formation and function has been provided by mice lacking Kalirin-7 (Kalirin-7 knockout mice) and mice lacking all Kalirin isoforms (*KALRN* knockout mice), both of which exhibit decreased spine density in the cortex and specific cognitive deficits (Cahill *et al.*, 2009; Ma *et al.*, 2008; Xie *et al.*, 2010). Interestingly, early stages of excitatory synapse development appear to proceed normally in these mice, suggesting that Kalirin-7, which is primarily expressed late in development and in the adult, may play a more essential role in spine maturation and/or maintenance (Cahill *et al.*, 2009; Ma *et al.*, 2008). Furthermore, reductions in Rac-GTP levels and spine density were only observed in the frontal cortex but not the hippocampus of adult *KALRN* knockout mice (Cahill *et al.*, 2009). Other Rac-GEFs such as Tiam1 and -PIX, which retain higher levels of expression in the adult hippocampus compared to the cortex, might partially compensate for the absence of Kalirin-7 in the hippocampus (Penzes *et al.*, 2008).

Kalirin-7 regulates spine dynamics downstream of a variety of synaptic receptors (Fig. 4). For instance, Kalirin-7 function is necessary for EphB-induced spine remodeling (Penzes *et al.*, 2003). EphB receptors belong to a large family of Eph receptor tyrosine kinases that play key roles in controlling spine morphogenesis and synapse development and plasticity (Klein, 2004; Yamaguchi and Pasquale, 2004; Klein, 2009). In response to stimulation by their membrane-bound ephrin ligands, EphB receptors promote the formation and maturation of spines (Ethell *et al.*, 2001; Henkemeyer *et al.*, 2003; Kayser *et al.*, 2006; Kayser *et al.*, 2008; Penzes *et al.*, 2003), whereas EphA receptors induce spine retraction (Fu *et al.*, 2007; Murai *et al.*, 2003). The ability of EphB receptors to stimulate spine morphogenesis and synapse maturation requires the activities of several Rho family GEFs (Irie and Yamaguchi, 2002; Penzes *et al.*, 2003; Tolias *et al.*, 2007). Kalirin-7 was shown to play a role in EphB receptor signaling by the demonstration that EphB receptor activation results in the phosphorylation and clustering of Kalirin-7 (Penzes *et al.*, 2003). Furthermore, expression of a GEF-dead Kalirin-7 mutant in cultured hippocampal neurons blocks spine development induced by ephrinB1 activation of EphB receptors (Penzes *et al.*, 2003). Taken

together, these results suggest that Kalirin-7 plays an important role in EphB-mediated spine morphogenesis.

As mentioned previously, neuronal activity exerts profound effects on the development and structural remodeling of dendritic spines (Engert and Bonhoeffer, 1999; Lendvai *et al.*, 2000; Maletic-Savatic *et al.*, 1999; Toni *et al.*, 1999). These activity-induced effects are mediated by the NMDA-type glutamate receptor, a calcium-permeable ion channel that plays a central role in synaptic plasticity and learning and memory formation (Cull-Candy *et al.*, 2001; MacDonald *et al.*, 2006; Yashiro and Philpot, 2008). Several Rac-specific GEFs have been implicated in activity-dependent signaling pathways that regulate the formation and remodeling of spines (Fig. 4) (Saneyoshi *et al.*, 2008; Tolias *et al.*, 2005; Xie *et al.*, 2007). In the case of Kalirin-7, NMDA receptor activation induces a CaMKII-dependent phosphorylation of Kalirin-7 on residue threonine 95, which was suggested to enhance its GEF activity in cultured neurons (Xie *et al.*, 2007). Overexpression of a Kalirin-7 non-phosphorylatable T95A mutant blocked NMDA receptor-dependent spine enlargement but not spine formation, suggesting that Kalirin-7 phosphorylation is necessary for activity-dependent increases in spine area (Xie *et al.*, 2007). Kalirin-7 also interacts with the GluR1 subunit of AMPA receptors, and RNAi knockdown of Kalirin-7 or deletion of all Kalirin isoforms prevented the NMDA receptor-induced spine enlargement and spine delivery of AMPA receptor typically observed in control cortical neuron cultures (Xie *et al.*, 2010; Xie *et al.*, 2007). Kalirin-7 knockout mice, however, have normal levels of GluR1 in purified PSDs, indicating that Kalirin-7 may not be essential for AMPA receptor localization *in vivo* (Ma *et al.*, 2008).

In addition to acting downstream of EphB and NMDA receptors, Kalirin-7 also regulates spine morphogenesis induced by N-cadherin clustering (Fig. 4). N-cadherin is a trans-synaptic adhesion molecule that plays an important role in regulating the morphology of dendritic spines and the formation, function, and plasticity of synapses (Bozdagi *et al.*, 2000; Bozdagi *et al.*, 2004; Okamura *et al.*, 2004; Takeichi, 2007; Tang *et al.*, 1998; Togashi *et al.*, 2002). N-cadherin interacts with Kalirin-7 via the scaffolding protein AF-6 (Xie *et al.*, 2008). Stimulation of cortical neuronal cultures with clustered N-cadherin results in an increase in spine size, which was blocked by RNAi knockdown of all Kalirin isoforms (Xie *et al.*, 2008). These results suggest that N-cadherin-mediated spine enlargement requires Kalirin.

As described in more detail below, Kalirin-7 is not the only Rac-GEF to localize to excitatory synapses and participate in EphB and NMDA receptor signaling pathways. Interestingly, however, its role is not compensated in the cortex by these other GEFs when Kalirin-7 function is lost. This observation suggests that Kalirin-7 plays a unique role at synapses in the cortex. One clue for determining Kalirin-7's distinct function at synapses comes from examining its differential expression. In contrast to other Rac-GEFs that are highly expressed during development, Kalirin-7 expression in the cortex and hippocampus begins around postnatal day (P) 10–15 and is retained at high levels throughout adulthood (Penzes *et al.*, 2008). Kalirin-7 may therefore play a more dominant role in spine maturation and maintenance rather than early spine formation. We propose that Kalirin-7 serves as a signal integrator that incorporates inputs from a variety of synaptic receptors late in development and induces Rac GTPase signaling pathways that favor spine maturation and remodeling. Its interaction with multiple PDZ domain-containing proteins may provide additional spatial specificity, possibly giving Kalirin-7-activated Rac access to a unique pool of actin within spines.

3.1.2. Tiam1—The Rac-specific GEF Tiam1 (T-lymphoma invasion and metastasis 1) also functions as a critical mediator of spine development (Tolias *et al.*, 2005; Tolias *et al.*, 2007;

Zhang and Macara, 2006). Tiam1 is a large multi-domain protein that consists of a myristoylation site, two PEST sequences, an N-terminal PH domain flanked by a coiled-coiled and an extended region (PHn-CC-Ex), a Ras-binding domain (RBD), a PDZ domain, and the characteristic DH-PH Rac-GEF domain (Fig. 3). The ability of Tiam1 to induce Rac-dependent actin remodeling and cell shape change requires both its Rac-GEF enzymatic activity and its ability to translocate to the plasma membrane, which is mediated by its PHn-CC-Ex domain (Mertens *et al.*, 2003). In *Drosophila*, the Tiam1 homolog Still life (SIF) localizes to synaptic terminals and plays a role in axonal extension and synaptic development (Sone *et al.*, 1997; Sone *et al.*, 2000). In mammals, Tiam1 is expressed at high levels in the developing brain, and its expression remains high in restricted adult brain regions including the hippocampus, the olfactory bulb and the cerebellum (Ehler *et al.*, 1997). Over the years, Tiam1 has been implicated in several neurodevelopmental processes including neuronal migration, neurite outgrowth, and axon specification (Kawauchi *et al.*, 2003; Kunda *et al.*, 2001; Leeuwen *et al.*, 1997). In addition, Tiam1 is present in dendrites and spines and localizes to the PSD (Tolias *et al.*, 2005). RNAi knockdown of Tiam1 expression in primary hippocampal and cortical neurons results in significant reductions in dendritic arborization and spine and synapse density, suggesting that Tiam1 is necessary for proper dendritic growth as well as spine and synapse development (Tolias *et al.*, 2005; Tolias *et al.*, 2007; Zhang and Macara, 2006).

Like Kalirin-7, Tiam1 functions in a number of signaling pathways (Fig. 4). For instance, Tiam1 interacts with the NMDA receptor and is phosphorylated in a calcium-dependent manner following NMDA receptor stimulation, leading to Rac activation (Tolias *et al.*, 2005). Calcium-dependent Tiam1 phosphorylation may be mediated by CaMKII, which can directly phosphorylate Tiam1 and enhance its GEF activity approximately 2-fold (Fleming *et al.*, 1999). In control cortical neurons, stimulation of the NMDA receptor leads to an increase in spine density (Tolias *et al.*, 2005). Notably, RNAi knockdown of Tiam1 prevents this increase in spine density, suggesting that Tiam1 is required for NMDA receptor-dependent spine formation (Tolias *et al.*, 2005). Furthermore, reduced Tiam1 expression blocks the NMDA receptor-stimulated phosphorylation and activation of AKT, a serine/threonine kinase that controls cell growth and actin cytoskeletal remodeling (Rodgers and Theibert, 2002; Tolias *et al.*, 2005). Together, these results suggest that Tiam1 helps link the NMDA receptor to activity-induced intracellular signaling pathways that regulate spine morphogenesis.

In addition to regulating NMDA receptor-induced spine development, Tiam1 mediates EphB receptor-dependent spine morphogenesis (Tolias *et al.*, 2007). Via its PHn-CC-Ex domain, Tiam1 specifically interacts with the EphB2 tyrosine kinase receptor in a kinase-dependent manner (Tolias *et al.*, 2007). Stimulation of EphB receptors with ephrinB induces the recruitment of Tiam1 to sites of new synaptic contacts and results in the phosphorylation of Tiam1 at tyrosine 829, promoting Rac1 activation (Miyamoto *et al.*, 2006; Tolias *et al.*, 2007). Importantly, disruption of Tiam1 function with RNAi knockdown or a dominant-negative Tiam1 mutant blocks ephrinB-induced spine formation in hippocampal neurons (Tolias *et al.*, 2007). Taken together, these results suggest that EphB receptors regulate spine development in part by recruiting, phosphorylating and activating Tiam1, which leads to Rac-dependent actin remodeling required for spine formation.

Finally, Tiam1 has been shown to cooperate with the partition-defective (PAR) protein PAR-3 in regulating spine morphogenesis (Zhang and Macara, 2006). PAR-3 is a member of the evolutionary conserved PAR polarity complex, consisting of PAR-3, PAR-6, and atypical protein kinase C (aPKCζ). The PAR complex PAR-3 controls many aspects of cell polarity, including asymmetric cell division, directional cell migration, epithelial apical-basal polarity, axon specification and synaptogenesis (Arimura and Kaibuchi, 2007;

Goldstein and Macara, 2007). Accumulating evidence indicates that Tiam1 and the closely related Tiam2/STEF are essential components of the PAR complex (Chen and Macara, 2005; Gerard *et al.*, 2007; Mertens *et al.*, 2005; Nishimura *et al.*, 2005; Pegtel *et al.*, 2007; Zhang and Macara, 2006). PAR-3 recruits Tiam1 or Tiam2 to the PAR complex, where they help to establish cell polarity by activating Rac and aPKCζ, resulting in polarized actin and microtubule reorganization (Mertens *et al.*, 2006). In the regulation of hippocampal dendritic spine morphogenesis, PAR-3 has been suggested to spatially restrict Tiam1 to spines, thereby preventing inappropriate Rac activation elsewhere (Zhang and Macara, 2006).

Tiam1, like Kalirin-7, interacts with many proteins and participates in a number of signaling pathways. Yet, several aspects of Tiam1's biology make it unique among synaptic Rac-GEFs. First, it interacts with the PAR complex, a crucial determinant of cell polarity. This interaction between Tiam1 and the PAR complex is thought to restrict Tiam1 activity in a polarized manner (Zhang and Macara, 2006), which could result in the modulation of a specific subpool of actin within the spine. Second, Tiam1 activates AKT, providing a link between synaptic receptors (NMDA receptor, EphB) and signaling pathways that control cell growth and cytoskeletal rearrangement (Tolias *et al.*, 2005). Third, Tiam1 interacts with GTP-bound Ras through its Ras-binding domain, and may therefore function as a Ras effector that mediates Rac-dependent synaptic remodeling in response to Ras activation (Lambert *et al.*, 2002; Malliri *et al.*, 2002). Finally, in addition to its role in dendritic development, Tiam1 regulates axonal differentiation and growth, and may therefore also contribute to presynaptic development, as is the case for its drosophila homolog SIF (Kunda *et al.*, 2001; Nishimura *et al.*, 2005; Sone *et al.*, 1997; Sone *et al.*, 2000).

3.1.3. β–PIX (Arhgef7)—The Pak-interacting exchange factor β-PIX has also been identified as a regulator of spine morphogenesis and synapse formation (Parnas *et al.*, 2001; Zhang *et al.*, 2003; Zhang *et al.*, 2005). β-PIX is a multidomain protein comprised of an SH3 domain, a DH-PH Rac/Cdc42 GEF domain, a GIT1-binding region, and a proline-rich region (Fig. 3) (Rosenberger and Kutsche, 2006). In the brain, two major β-PIX isoforms are expressed that differ only at their C-termini; β1-PIX possesses a coiled-coil dimerization domain and a PDZ-binding motif, whereas β2-PIX contains a serine-rich region (Koh *et al.*, 2001). These two β-PIX isoforms are present at high levels during development, and they continue to be expressed in the adult in restricted brain regions such as the hippocampus and cerebellum (Kim *et al.*, 2000). β-PIX was originally identified based on its ability to interact via its SH3 domain with the serine/threonine kinase Pak, a major downstream effector of Rac and Cdc42 (Bagrodia *et al.*, 1998; Manser *et al.*, 1998). By binding to Pak and activating Rac and/or Cdc42, β-PIX helps to coordinate Rac/Cdc42-dependent Pak activation, which promotes actin cytoskeletal remodeling (Manabe *et al.*, 2002; Zhang *et al.*, 2005). Interestingly, activated Pak can also phosphorylate β-PIX on residue threonine 526, increasing β-PIX's membrane localization and enhancing its GEF activity (Shin *et al.*, 2002). This result suggests that the β-PIX/Rac/Cdc42/Pak signaling cascade is regulated by a positive feedback loop.

In hippocampal neurons, β-PIX localizes to synapses and is targeted to the PSD through its interaction with the synaptic scaffolding protein GIT1 (G protein-coupled receptor kinase-interacting protein 1) (Collins *et al.*, 2006; Zhang *et al.*, 2003; Zhang *et al.*, 2005). GIT1 is a ubiquitously expressed Arf-GAP that forms a signaling complex with β-PIX and Pak (Manabe *et al.*, 2002). Disruption of GIT function results in β-PIX mislocalization and a decrease in spine and synapse density, suggesting that GIT1 regulates spine morphogenesis and synapse formation by recruiting β-PIX to synapses and restricting Rac activation (Zhang *et al.*, 2003; Zhang *et al.*, 2005). Localized Rac activation, in turn, induces Pak activation and Pak-mediated myosin light chain (MLC) phosphorylation, which are both required for

proper spine formation (Zhang *et al.*, 2005). By interacting with additional pre- and post-synaptic proteins including Shank, Piccolo and liprin-α, β-PIX and GIT1 appear to regulate active zone cytoskeletal matrix organization and AMPA receptor targeting (Kim *et al.*, 2003; Ko *et al.*, 2003; Park *et al.*, 2003). Consistent with these findings, the *Drosophila* homolog of β-PIX, dPIX, regulates post-synaptic structure and protein localization at the *Drosophila* glutamatergic neuromuscular junctions (Parnas *et al.*, 2001).

Like Kalirin-7 and Tiam1, β-PIX functions downstream of synaptic receptors (Fig. 4). For instance, NMDA receptor stimulation induces the activation of calmodulin-dependent kinase kinase (CaMKK) and CaMKI, which form a multiprotein complex with β-PIX and GIT1 in spines (Saneyoshi *et al.*, 2008). CaMKI-mediated phosphorylation of serine 516 in β-PIX enhances its GEF activity, resulting in the activation of Rac. Blocking this pathway in the hippocampus with pharmacological inhibitors, dominant-negative constructs or RNAi reduces spine formation and miniature excitatory postsynaptic current (mEPSC) frequency, which can be rescued by constitutively active Pak (Saneyoshi *et al.*, 2008). These results suggest a role for β-PIX in calcium-calmodulin signaling cascades that regulate spine morphogenesis. Furthermore, GIT1 was also recently implicated in spine morphogenesis induced by ephrinB reverse signaling (Segura *et al.*, 2007). EphrinB activation results in the phosphorylation of GIT1 on tyrosine 392, creating a docking site for the adaptor protein Grb4, which binds to the tail of activated ephrinB. Disrupting the ephrinB-Grb4-GIT1 complex blocks reverse signaling and impairs normal spine morphogenesis and synapse formation (Saneyoshi *et al.*, 2008).

Though it shares some similarities with Kalirin-7 and Tiam1, β-PIX has several unique features that may explain why these other Rac-GEFs are unable to fully compensate for its loss. Particularly striking is its interactions with Pak and GIT1 and its putative positive feedback loop. These features suggest both a temporally explosive all-or-none activation of its GEF activity and tight regulation of its signal in space.

3.1.4. Intersectin-L (Itsn1)—Intersectin is a multidomian scaffolding protein that is best known for its role in regulating endocytosis in non-neuronal cells and in synaptic vesicle recycling at the neuromuscular junction (NMJ) in *Drosophila* and *C. elegans* (Koh *et al.*, 2004; Marie *et al.*, 2004; Rose *et al.*, 2007; Wang *et al.*, 2008). Intersectin possesses two N-terminal Eps15 homology domains, several coiled-coil domains, and five SH3 domains (Yamabhai *et al.*, 1998) (Fig. 3). In vertebrates, the Intersectin gene is subject to alternative splicing, resulting in the generation of a longer isoform (Intersectin-L) that is expressed exclusively in neurons (Pucharcos *et al.*, 1999). In addition to the domains it shares with the shorter, ubiquitously expressed Intersectin-S isoform, Intersectin-L also contains a C-terminal C2 domain and a DH-PH domain with GEF activity specific for Cdc42 (Hussain *et al.*, 2001; Snyder *et al.*, 2002). Not only does Intersectin-L activate Cdc42, it also interacts with a number of actin regulatory proteins, suggesting a role in actin cytoskeletal regulation (Hussain *et al.*, 2001). Indeed, Intersectin-L can induce filopodia formation when overexpressed in fibroblast cells (Hussain *et al.*, 2001). By controlling both actin remodeling and the recruitment of endocytic proteins such as dynamin1 and synaptojanin1 (Koh *et al.*, 2004; Marie *et al.*, 2004), Intersectin-L may function to couple membrane trafficking with actin cytoskeletal remodeling.

Full length Intersectin-L exhibits little GEF activity and appears to be maintained in an auto-inhibited conformation by an intramolecular interaction between its SH3 and DH domains, which blocks Cdc42 binding (Zamanian and Kelly, 2003). N-WASP (neuronal Wiskott-Aldrich syndrome protein), a critical regulator of Arp2/3-mediated actin polymerization, interacts directly with the SH3 domains of Intersectin-L and relieves this autoinhibition, resulting in Cdc42 activation (Hussain *et al.*, 2001). The EphB2 receptor also interacts with

Intersectin-L and stimulates its GEF activity in cooperation with N-WASP (Irie and Yamaguchi, 2002). Cdc42 activation by Intersectin-L then leads to N-WASP activation and the stimulation of actin polymerization (Hussain *et al.*, 2001). Importantly, in hippocampal neurons Intersectin-L co-localizes with dendritic spine F-actin, and RNAi knockdown of Intersectin-L or disruption of the interaction between Intersectin-L and N-WASP perturbs spine development, resulting in an increase in filopodia-like protrusions and a decrease in mushroom-shaped spines (Irie and Yamaguchi, 2002; Nishimura *et al.*, 2006; Thomas *et al.*, 2009). Taken together, these results suggest that Intersectin-L plays an important role in spine maturation by activating Cdc42 and N-WASP, leading to actin cytoskeletal remodeling and possibly membrane trafficking (Fig. 4). Though it is likely that other Cdc42 GEFs participate in synaptogenesis, L-intersectin's potential coupling of actin dynamics and membrane trafficking and its distinctive relationship with N-WASP provide important clues to its unique role at synapses. Given the plethora of protein interaction domains that L-intersectin contains, identification of additional binding partners will be required to elucidate its particular function.

3.2 RhoA-GEFs at excitatory synapses

3.2.1. Lfc (GEF-H1, Arhgef2)—Rho-specific GEFs such as Lfc have also been implicated in the formation and structural remodeling of synapses (Kang *et al.*, 2009; Ryan *et al.*, 2005). Lfc is a Rho-GEF that is highly expressed in the brain (Ryan *et al.*, 2005). Its domain structure includes a C1 domain, a DH-PH domain and a coiled-coil region (Fig 3). Under basal conditions, Lfc is mainly restricted to the dendritic shafts in cultured hippocampal neurons, likely through its association with microtubules (Glaven *et al.*, 1999; Ren *et al.*, 1998; Ryan *et al.*, 2005). NMDA receptor stimulation results in the rapid translocation of Lfc into dendritic spines (Muly *et al.*, 2008; Ryan *et al.*, 2005). In spines, Lfc is present in the PSD (Collins *et al.*, 2006; Kang *et al.*, 2009), where it interacts with AMPA receptors (Kang *et al.*, 2009) as well as with Spinophilin and Neurabin, two homologous proteins that associate with F-actin (Fig. 5) (Ryan *et al.*, 2005). The recruitment of Lfc into spines likely results in RhoA activation, leading to actin cytoskeletal remodeling and alterations in spine morphology. Indeed, overexpression of Lfc in cultured hippocampal neurons was shown to reduce spine length and size (Ryan *et al.*, 2005), whereas blocking Lfc function with dominant-negative mutants or RNAi results in an increase in spine size and density (Kang *et al.*, 2009; Ryan *et al.*, 2005). Furthermore, AMPA receptor-dependent spine remodeling appears to require Lfc (Kang *et al.*, 2009). Treatment of hippocampal neurons with an AMPA receptor antagonist normally causes a decrease in spine density as well as an increase in RhoA activation. These effects were ablated in neurons in which Lfc function was blocked (Kang *et al.*, 2009). Together, these results indicate that Lfc plays a critical role in RhoA-mediated spine retraction. It is interesting to speculate that Lfc is normally restricted to dendritic shafts to allow for new spine growth during early synapse development. Furthermore, following NMDA receptor activation, Lfc-mediated RhoA activation may be intentionally time-delayed from the onset of an activity-induced signal due to the need of Lfc to translocate from the dendritic shaft into spines. In spines, Lfc's specificity would be further enhanced by its interaction with AMPA receptors and specific F-actin binding proteins.

3.2.2. Ephexin1/5 (Ngef/Vsm-RhoGEF)—Ephexins are another family of Rho-GEFs implicated in synapse regulation (Frank *et al.*, 2009; Fu *et al.*, 2007; Margolis *et al.*; Shi *et al.*, 2010). The Ephexin family consists of five members (Ephexin1 through Ephexin5), each encoded by a different gene. Ephexin family members share the same overall structure, including a single DH-PH domain and a C-terminal SH3 domain, but possess unique N-terminal regions (Fig. 3) (Sahin *et al.*, 2005). Of the five family members, only Ephexin1 and Ephexin5 are highly expressed in the developing nervous system (Sahin *et al.*, 2005).

Ephexin1 was originally identified based on its ability to interact with the cytoplasmic domain of the EphA4 receptor (Shamah *et al.*, 2001). When overexpressed in cells, Ephexin1 can activate RhoA, Cdc42 and Rac, but its GEF activity towards RhoA appears to be specifically enhanced following EphA4-induced tyrosine phosphorylation (Sahin *et al.*, 2005; Shamah *et al.*, 2001). Ephexin1 is best known for its role in mediating ephrinA-stimulated growth cone collapse (Sahin *et al.*, 2005; Shamah *et al.*, 2001). Recently, however, Ephexin1 has also been implicated in regulating different aspects of synapse development and function. For instance, EphA4-mediated spine retraction in the hippocampus has been shown to require both Ephexin1 and the serine/threonine kinase Cdk5 (Fu *et al.*, 2007). EphA4 receptor activation causes the recruitment of Cdk5 to EphA4, resulting in Cdk5 phosphorylation and activation (Fu *et al.*, 2007). Activated EphA4 and Cdk5 then induce the recruitment and phosphorylation of Ephexin1, leading to RhoA activation and reduced spine density (Fig 5) (Fu *et al.*, 2007). In addition to mediating EphA4-induced spine retraction, Ephexin1 also plays an essential role in regulating presynaptic homeostatic signaling in *Drosophila* (Frank *et al.*, 2009) and the structural maturation and neurotransmission of NMJs in mice (Shi *et al.*, 2010), which will be discussed in more detail below.

Recently, Ephexin5 was also identified as a negative regulator of excitatory synapse development (Margolis *et al.*, 2010). RNAi knockdown or genetic ablation of Ephexin5 increases dendritic spine and synapse density in hippocampal neurons, whereas overexpression of Ephexin5 decreases excitatory synapse number by selectively activating RhoA (Margolis *et al.*, 2010). Ephexin5 preferentially binds to EphB2, and activation of EphB2 triggers the phosphorylation, ubiquitination, and degradation of Ephexin5, resulting in EphB-dependent excitatory synapse development (Margolis *et al.*, 2010). The degradation of Ephexin5 is mediated by the ubiquitin ligase Ube3A (Margolis *et al.*, 2010), which is mutated in the human cognitive disorder Angelman syndrome and is duplicated in some forms of Autism Spectrum Disorders (ASDs) (Dan, 2009). These findings suggest that Ephexin5 restricts synapse formation by activating RhoA, and that this suppression is relieved by EphB receptor activation during synapse development (Fig. 5). Disruption of EphB-mediated Ephexin5 degradation in the absence of Ube3A function may contribute to the cognitive and synaptic defects associated with Angelman syndrome.

4. Rho-family GEF involved in inhibitory synapse development

Although much of the research in this field has been directed toward the formation of excitatory synapses, Rho GTPase regulatory proteins also function in the formation of other types of synapses. In this section, we will summarize briefly the specific functions of a Rho-family GEF known to participate in the formation of inhibitory synapses.

4.1. Collybistin (Arhgef9)

Collybistin, encoded by rodent *ARHGEF9*, is by far the best-characterized Rho-family GEF involved in the formation of inhibitory synapses. Collybistin contains an N-terminal SH3 domain, a single DH-PH domain with Cdc42 GEF activity, and a C-terminal coiled-coil domain (Fig. 3). Collybistin knockout mice exhibit a striking loss of inhibitory synapses, both morphologically and functionally (Jedlicka *et al.*, 2009; Papadopoulos *et al.*, 2008; Papadopoulos *et al.*, 2007). These defects are accompanied by increased anxiety and reduced spatial learning (Papadopoulos *et al.*, 2007). Collybistin promotes the formation of inhibitory synapses by clustering gephryn, a protein scaffold that, in turn, clusters glycine and GABA receptors (Harvey *et al.*, 2004; Kins *et al.*, 2000). Collybistin expression is upregulated between embryonic day (E) 7–11, when the postmitotic neurons in which it is primarily expressed begin to arise (Kneussel *et al.*, 2001). In addition to its critical interaction with gephyrin, collybistin's N-terminal domain interacts with neuroligin-2

(Poulopoulos *et al.*, 2009). This interaction, without which full length collybistin cannot cluster gephyrin, is crucial for the formation of inhibitory synapses *in vivo* (Poulopoulos *et al.*, 2009).

Interestingly, a question remains as to whether or not collybistin functions as a GEF in the formation of inhibitory synapses. The human homologue of collybistin, h-PEM2, functions *in vitro* as a GEF for Cdc42, but not for Rac1 or RhoA (Reid *et al.*, 1999). Using a number of collybistin point mutants that are unable to activate Cdc42, Reddy-Alla *et al.* (Reddy-Alla *et al.*, 2010) showed that collybistin's Cdc42 GEF activity and, indeed, Cdc42, are dispensable for clustering gephyrin, and, presumably, for inhibitory synapse formation. These results suggest the possibility that collybistin's GEF activity towards Cdc42 is not required for inhibitory synapse formation. However, it remains to be seen whether the synapses that form with mutant collybistin or in the absence of Cdc42 are functional. It is also not clear whether collybistin activates other Rho-family GTPases, and, if so, whether or not the collybistin mutants used by Reddy-Alla et al. are inactive toward these GTPases. Further investigation will clarify these issues. In sum, collybistin occupies a pivotal location in the protein interactome linking neuroligin-2, which can detect juxtacellular signals, to gephyrin, the scaffold on which inhibitory synapses are built. This location implies a role for collybistin in linking signals to inhibitory synapse formation, though it is presently unclear whether its GEF function is required.

5. Rho-family GEFs involved in neuromuscular junction development

In this section, we will discuss two Rho-family GEFs that play a role in NMJ development. As above, we will consider the regulation and connectivity of these proteins in order to propose a specific niche for each.

5.1. Trio

Although the Rho-family GEF Trio is best known for its role in axon guidance (Bateman and Van Vactor, 2001), recent studies of the Trio homolog UNC-73 in *C. elegans* have indicated a potential role in synaptogenesis as well. Trio and UNC-73 are large proteins closely related to Kalirin that possess numerous domains including a Sec14p domain, multiple spectrin-like repeats, an SH3 domain, and two DH-PH Rho GEF domains (RhoGEF-1 specific for Rac1/RhoG and RhoGEF-2 specific for RhoA) (Fig. 3). Full length mammalian Trio, containing an additional SH3 domain, an Ig/fibronectin-like domain (Ig/FN) and a kinase domain, is expressed in skeletal muscle, whereas shorter forms of Trio lacking these domains are enriched in the brain (McPherson *et al.*, 2005). Mice lacking Trio exhibit skeletal muscle defects and aberrant organization of several brain regions (O'Brien *et al.*, 2000). In *Drosophila* and *C. elegans*, Trio/UNC-73 functions in axon guidance by activating Rac (via its RhoGEF-1 domain), resulting in actin cytoskeletal reorganization necessary for growth cone guidance and outgrowth (Awasaki *et al.*, 2000; Bateman *et al.*, 2000; Forsthoefel *et al.*, 2005; Newsome *et al.*, 2000; Steven *et al.*, 1998; Vanderzalm *et al.*, 2009). Interestingly, mutations restricted to RhoGEF-2 domain of UNC-73 cause early stage lethality and musculature and neurotramission defects in the absence of axon guidance defects, suggesting additional non-guidance functions for UNC-73 that require the RhoGEF-2 domain (Steven *et al.*, 2005). UNC-73 was also independently shown to be necessary for proper extension of the muscle arm, a step involved in the formation of the NMJ (Alexander *et al.*, 2009). Again, UNC-73's GEF activity was required for this function, though this study implicated UNC-73's RhoGEF-1 domain (Alexander *et al.*, 2009). Taken together, these data suggest that UNC-73/Trio functions as a GEF in NMJ formation, with the RhoGEF-1 domain being of primary importance. UNC-73 also signals through its RhoGEF-2 domain to regulate musculature and synaptic neurotransmission (Steven *et al.*, 2005), though the relevance of this to NJM formation is not known.

Defining a specific role for UNC-73/Trio in NMJ formation is not yet possible. Though full length mammalian Trio is present in muscle, UNC-73 lacks an equivalent serine/threonine kinase-domain, suggesting that this activity is not required for the functions described here. It is known that UNC-73 localizes to the ends of the muscle arm, a postsynaptic intermediate in NMJ formation (Alexander *et al.*, 2009). There it colocalizes with the transmembrane receptor UNC-40/Dcc, which functions upstream of UNC-73 in NMJ formation (Alexander *et al.*, 2009). UNC-73/Trio also regulates diaphanous-related formins presynaptically in NMJ formation (Pawson *et al.*, 2008). However, the signals that control UNC-73/Trio activity in NMJ formation are not known, nor are whether its actions are due to its ability to activate Rac, RhoG (Estrach *et al.*, 2002), neither, or both. The actin-regulatory WAVE complex functions with UNC-40/73 in NMJ formation, suggesting a role for Rac in this process (Alexander *et al.*, 2009). Axon guidance studies have identified addition UNC-73/Trio-interacting proteins that may also function in NMJ formation, including CRML-1 (Vanderzalm *et al.*, 2009), an inhibitor of Trio's GEF activity, and the *Drosophila* netrin receptor Frazzled (Forsthoefel *et al.*, 2005).

5.2. Ephexin1 (Ngef)

In addition to regulating hippocampal excitatory synapse elimination (described above), Ephexin1 participates in the maturation of NMJs. Ephexin-1 knockout mice have weak forelimbs and perform poorly in the rotarod test (Shi *et al.*, 2010). NMJ transmission is decreased in these animals, and severe morphological defects in NMJ are apparent, though contacts between nerve and muscle do form (Shi *et al.*, 2010). Postsynaptic (muscular) clusters of Acetylcholine (Ach) receptors normally undergo a marked rearrangement from an oval morphology to a more complex "pretzel-like" morphology after development of the NMJ. This fails to occur in the Ephexin-1 knockout animals (Shi *et al.*, 2010). Addition of functional Ephexin-1 or constitutively active RhoA allows this transformation to proceed and NMJ development to reach completion (Shi *et al.*, 2010), suggesting a role for Ephexin-1-mediated RhoA activation in controlling the distribution of Ach receptors in developing muscle.

While it is not yet demonstrated what the signal upstream of Ephexin-1 in NMJ maturation is, Shi et al. (Shi *et al.*, 2010) speculate that it comes from EphA4. As in growth cone collapse and spine elimination, phosphorylation of ephexin-1 on tyrosine-87 is required for its activation (Fu *et al.*, 2007; Shi *et al.*, 2010). This result suggests that Ephexin-1 is a multifunctional GEF that ties ephrin-A/EphA signals to a variety of neuronal phenomena through RhoA-mediated cytoskeletal changes. It will be interesting to determine whether Cdk5 is also a common theme in Ephexin-1 signaling and whether Ephexin-1 has other partners whose identity can help us to better understand its precise role in these differing phenomena.

6. Rho-family GAPs involved in excitatory synaptogenesis

Less is known about the role of Rho GTPase GAPs in synaptogenesis, since historically they have been viewed as signal terminators with a secondary role in comparison with the Rho GTPase GEFs, which activate Rho GTPases in response to diverse extracellular stimuli (Tcherkezian and Lamarche-Vane, 2007). It is becoming increasingly clear, however, that GAPs are also highly regulated proteins that play essential roles in controlling specific Rho GTPase-mediated cellular processes such as synaptogenesis. Here we will discuss the function and regulatory mechanisms of several Rho GTPase GAPs that have recently been implicated in synapse development, again highlighting the specific roles that they play.

6.1. Rac/Cdc42-GAPs at excitatory synapses

6.1.1. α 1-chimaerin—The diacyglyerol (DAG)-binding protein α1-chimaerin was one of the first Rho-family GAPs implicated in regulating dendritic spine morphogenesis (Buttery *et al.*, 2006; Van de Ven *et al.*, 2005). α1-chimaerin belongs to the chimaerin family of Rho GAPs that also includes α2-, β1- and β2-chimaerin. These chimaerin isoforms are generated as alternatively spliced products from the α- and β-chimaerin genes. All four chimaerin proteins possess a C1 phorbol ester- and DAG-binding domain and a Rho GAP domain that specifically inhibits Rac (Ahmed *et al.*, 1993; Caloca *et al.*, 2003; Diekmann *et al.*, 1991). α2- and β2-chimaerin also have an additional N-terminal SH2 (Src Homology 2) domain capable of binding to phosphotyrosine-containing residues (Leung *et al.*, 1994) (Fig. 3). Unlike the other chimaerin isoforms, α1-chimaerin is exclusively expressed in the brain, and its expression is upregulated during synaptogenesis in response to neuronal activity (Buttery *et al.*, 2006; Diaz *et al.*, 2002; Lim *et al.*, 1992).

In hippocampal neurons, α1-chimaerin is present in dendrites and spines (Van de Ven *et al.*, 2005). Overexpression of α1-chimaerin causes loss of spines and a simplification of the dendritic arbor that requires both the DAG-binding site and the Rac-GAP activity of α1-chimaerin (Buttery *et al.*, 2006; Van de Ven *et al.*, 2005). In contrast, overexpression of α2-chimaerin induces process outgrowth rather than dendritic pruning, suggesting a differential role for the SH2 domain in α2-chimaerin (Buttery *et al.*, 2006). To further investigate the role of α1-chimaerin in regulating dendritic morphogenesis, two independent groups performed RNAi experiments in hippocampal neurons, with slightly different results. Van de Ven et al. (Van de Ven *et al.*, 2005) reported that α1-chimaerin suppression increases spine density, whereas Buttery et al. (Buttery *et al.*, 2006) demonstrated that α1-chimaerin silencing leads to excess outgrowth from spine heads and dendrites, resulting in a greater number of atypical spines and filopodia-like protrusions. Taken together, these results suggest that -chimaerin plays an important role in restricting dendritic protrusions in hippocampal neurons by inhibiting Rac signaling.

One of the distinguishing features of chimaerin proteins is their C1 domain, which binds to phorbol esters and DAG and regulates the recruitment of chimaerins to the plasma membrane (Ahmed *et al.*, 1990; Caloca *et al.*, 2001). In hippocampal neurons, stimulation of phospholipase Cβ (PLCβ)-coupled cell surface receptors that induce DAG production results in the rapid translocation of α1-chimaerin to the plasma membrane (Buttery *et al.*, 2006). The ability of α1-chimaerin to bind DAG is not only necessary for this translocation but also for the pruning activity of α1-chimaerin (Buttery *et al.*, 2006). α1-chimaerin also binds to the NR2A subunit of NMDA receptor in a phorbol ester-dependent manner, and this interaction is similarly required for α1-chimaerin's ability to regulate spine density (Van de Ven *et al.*, 2005). DAG signaling may therefore initiate α1-chimaerin recruitment to synaptic NMDA receptors, resulting in local inactivation of Rac1 and the pruning of dendritic protrusions (Fig. 4). Since α1-chimaerin continues to be expressed in adult neurons (Lim *et al.*, 1992), it is possible that α1-chimaerin also contributes to the ongoing remodeling of synapses in response to environmental stimuli and synaptic activity, but verification of this possibility awaits further investigation.

6.1.2. Bcr/Abr—Recently, another family of Rac-GAPs consisting of Bcr (Breakpoint cluster region) and Abr (active Bcr-related) has been implicated in excitatory synapse regulation in the hippocampus (Oh *et al.*, 2010). Bcr is best known for its involvement in Philadelphia chromosome-positive chronic myelogenous leukemia (Groffen and Heisterkamp, 1997). However, Bcr and Abr are highly expressed in the CNS (Fioretos *et al.*, 1995; Tan *et al.*, 1993), and mutant mice lacking both proteins exhibit cerebellar developmental defects, indicating a role for these proteins in nervous system development

(Kaartinen *et al.*, 2001). Bcr and Abr both contain a Rho GAP domain (Fig. 3) and function as potent inhibitors of Rac *in vitro* and *in vivo* (Cho *et al.*, 2007; Chuang *et al.*, 1995; Diekmann *et al.*, 1991; Kaartinen *et al.*, 2001; Tan *et al.*, 1993; Voncken *et al.*, 1995). In addition to their GAP domains, Bcr and Abr possess a number of additional signaling domains including a C2 domain, a PDZ-binding motif, and a DH-PH GEF domain that modestly activates Cdc42 and RhoA (Korus *et al.*, 2002; Malmberg *et al.*, 2004; Oh *et al.*, 2010; Radziwill *et al.*, 2003; Rizo and Sudhof, 1998; Sahay *et al.*, 2008). Bcr also contains an N-terminal coiled-coil oligomerization domain (McWhirter *et al.*, 1993) and a serine/threonine protein kinase domain that phosphorylates the adaptor proteins AF-6 and 14-3-3 (Fig. 3) (Li and Smithgall, 1996; Maru and Witte, 1991; Radziwill *et al.*, 2003).

Despite the fact that Bcr and Abr are abundantly expressed in the brain, relatively little is known about their function in neurons. In a recent report, Bcr and Abr were shown to localize to excitatory synapses and directly interact with PSD-95 (Oh *et al.*, 2010). Mice lacking Bcr or Abr exhibit a decrease in the maintenance, but not the induction, of LTP, and display impaired spatial learning and object recognition (Oh *et al.*, 2010). Bcr and Abr knockout mice also show a small increase in hippocampal neuron spine density compared to wild-type mice, but no difference in synaptic transmission (Oh *et al.*, 2010). These results indicate that Bcr and Abr play important roles in regulating synaptic plasticity and learning and memory, and suggest that excessive Rac activity hinders synaptic and cognitive function. Since Bcr and Abr have been demonstrated to compensate for each other's functions *in vivo* (Cho *et al.*, 2007; Kaartinen *et al.*, 2001; Kaartinen *et al.*, 2002), it will be interesting to determine if mice lacking both proteins have more significant defects in synapse development or function.

6.2. RhoA-GAPs at excitatory synapses

6.2.1. Oligophrenin-1—The Rho-GAP Oligophrenin-1 has also emerged as an important regulator of synapse development (Govek *et al.*, 2004; Khelfaoui *et al.*, 2007; Khelfaoui *et al.*, 2009; Nadif Kasri *et al.*, 2009). Oligophrenin-1 is encoded by *OPHN1*, which was first identified as an X-linked mental retardation gene (Billuart *et al.*, 1998). It is abundantly expressed in the nervous system during development and at later stages in highly plastic brain regions, such as the olfactory bulb and hippocampus (Fauchereau *et al.*, 2003). Oligophrenin-1 possesses several domains including an N-terminal membrane deforming Bin/Amphiphysin/Rvs (BAR) domain, a lipid binding PH domain, a Rho GAP domain that negatively regulates RhoA, Rac1 and Cdc42, and three proline-rich regions at its C-terminus (Billuart *et al.*, 1998; Fauchereau *et al.*, 2003; Govek *et al.*, 2004; Khelfaoui *et al.*, 2009) (Fig. 3).

Oligophrenin-1 is localized at pre- and postsynaptic sites in hippocampal neurons and has been implicated in regulating spine morphogenesis (Govek *et al.*, 2004). Knocking down Oligophrenin-1 in CA1 neurons from rat hippocampal slices significantly reduces the length of spines (Govek *et al.*, 2004). This phenotype was largely rescued by inhibiting Rho-kinase activity using the pharmacological inhibitor Y-27632, suggesting that the RhoA/Rho-kinase signaling pathway mediates the effect of Oligophrenin-1 knockdown on spine length. These results indicate that Oligophrenin-1 normally maintains spine length by repressing the RhoA/Rho-kinase pathway. Suppression of Oligophrenin-1 expression would therefore relieve RhoA inhibition, resulting in Rho-kinase activation and actin cytoskeletal remodeling that promotes spine shortening. Consistent with this idea, Oligophrenin-1 mutant mice display spine abnormalities and altered pre-synaptic function as well as behavioral, social, and cognitive impairments (Khelfaoui *et al.*, 2007).

Recently, Nadif Kasri *et al.* (Nadif Kasri *et al.*, 2009) demonstrated that overexpression of Oligophrenin-1 in hippocampal neurons selectively enhances AMPA receptor-mediated

synaptic transmission and increases spine size (Nadif Kasri *et al.*, 2009). Conversely, they showed that reduction of Oligophrenin-1 expression inhibits AMPA receptor- and NMDA receptor-mediated currents and hinders synaptic maturation, LTP and structural plasticity (Nadif Kasri *et al.*, 2009). These defects were rescued by reintroduction of wild-type Oligophrenin but not a mutant that lacks Rho-GAP activity, suggesting that Oligophrenin-1 controls AMPA receptor-mediated transmission and spine structure by repressing RhoA activity (Nadif Kasri *et al.*, 2009). Interestingly, Oligophrenin-1 was also shown to translocate into spines in response to neuronal activity and associate with AMPA receptor complexes (Nadif Kasri *et al.*, 2009). Using a peptide derived from the C-terminus of the AMPA receptor GluR2 subunit that blocks AMPA receptor endocytosis (Ahmadian *et al.*, 2004), Nadif Kasri *et al.* (Nadif Kasri *et al.*, 2009) demonstrated that Oligophrenin-1 regulates synaptic function and structure by stabilizing synaptic AMPA receptors.

Khelfaoui *et al.* (Khelfaoui *et al.*, 2009) have also recently provided evidence indicating a role for Oligophrenin-1 in regulating AMPA receptor trafficking in the hippocampus. They showed that Oligophrenin-1 is recruited to endocytic sites by interacting with three SH3 domain-containing adaptor proteins involved in clathrin-mediated endocytosis: amphiphysins, endophilins and CIN85 (Khelfaoui *et al.*, 2009). Furthermore, disruption of *OPHN1* in mice was shown to reduce endocytosis of synaptic vesicles at presynaptic sites and AMPA receptor internalization at postsynaptic sites, resulting in a significant impairment in NMDA receptor-dependent LTD (Khelfaoui *et al.*, 2009). Importantly, pharmacological inhibition of the RhoA/Rho-kinase pathway fully rescued the endocytosis and LTD defects caused by loss of Oligophrenin-1, indicating that Oligophrenin-1 normally regulates these processes by repressing RhoA/Rho-kinase signaling. Additionally, Nakano-Kobaya *et al.* (Nakano-Kobayashi *et al.*, 2009) demonstrated that Oligophrenin-1 controls synaptic vesicle cycling by forming a complex with the endocytic regulatory protein endophilin A1. Taken together, these results indicate that Oligophrenin-1 plays a critical role in regulating excitatory synapse morphogenesis and function by inhibiting RhoA-dependent signaling pathways that control both actin cytoskeletal remodeling and membrane trafficking at synapses.

6.2.2. p190 RhoGAP—p190 RhoGAP was first identified as a p120 RasGAP-interacting protein in Src-transformed cells (Ellis *et al.*, 1990). p190 RhoGAP contains an N-terminal GTP-binding domain, four consecutive FF protein-interaction domains, several proline-rich regions, and a C-terminal GAP domain with specific activity towards RhoA (Ridley *et al.*, 1993) (Fig. 3). p190 RhoGAP is a major substrate of the tyrosine kinase Src in the brain, and phosphorylation of p190 by Src enhances its interaction with p120 RasGAP and inhibits its RhoA-GAP and GTP-binding activities (Roof *et al.*, 2000). p190 RhoGAP is highly expressed in the developing nervous system, and mice lacking functional p190 RhoGAP exhibit neural developmental defects including abnormalities in forebrain hemisphere fusion and neural tube closure (Brouns *et al.*, 2000). p190 RhoGAP mutant mice also display aberrations in axon outgrowth, guidance and fasciculation, suggesting a role for p190 RhoGAP in axonal development (Brouns *et al.*, 2001). This role appears to be conserved in *Drosophila*, since RNAi knockdown of p190 RhoGAP expression in *Drosophila* causes retraction of axonal branches by up-regulating RhoA signaling, resulting in actin/myosin contractility (Billuart *et al.*, 2001).

In addition to regulating axonal development, p190 RhoGAP localizes to spines and promotes hippocampal neuron dendritic spine maturation and synapse and dendrite stability by inhibiting RhoA activity during late postnatal development (Sfakianos *et al.*, 2007; Zhang and Macara, 2008). The function of p190 RhoGAP at synapses is regulated by the tyrosine kinase Arg, which phosphorylates p190 RhoGAP and promotes its binding to p120 RasGAP, resulting in p190 membrane recruitment and RhoA inactivation (Bradley *et al.*,

2006; Sfakianos *et al.*, 2007). Mice lacking Arg display reduced p190RhoGAP phosphorylation, increased RhoA activity, progressive loss of synapses and dendritic branches, and deficits in a hippocampal-dependent novel object recognition task (Sfakianos *et al.*, 2007). *p190 RhoGAP* mutations enhance the effects of *arg* mutations on dendritic arborization, whereas mutations in the Rho-kinase ROCKII suppress the dendritic regression phenotype observed in the Arg knockout mice (Sfakianos *et al.*, 2007). These results indicate that Arg and p190 RhoGAP function together in hippocampal neurons during synaptic refinement to promote spine maturation and synapse and dendrite stability by inhibiting RhoA activity.

p190 RhoGAP has also been implicated in regulating spine morphogenesis in cooperation with members of the PAR-3/PAR-6/aPKC polarity complex in the hippocampus (Zhang and Macara, 2008) (Fig. 5). As mentioned previously, the polarity protein PAR-3 controls spine development by spatially restricting the Rac-GEF Tiam1 to dendritic spines, resulting in local Rac1 activation (Zhang and Macara, 2006). Surprisingly, this ability of PAR-3 to regulate spine morphogenesis does not appear to require the other members of the PAR complex (Zhang and Macara, 2006). Instead, PAR-6 and aPKC were shown to regulate spine formation and maintenance by inhibiting RhoA activity via p190 RhoGAP (Zhang and Macara, 2008). Specifically, a dominant-negative RhoA mutant and a Rho-kinase inhibitor were demonstrated to rescue the loss of spine formation caused by silencing PAR-6 expression, whereas a GAP-deficient p190 mutant inhibited the increase in spine density caused by PAR-6 overexpression (Zhang and Macara, 2008). RNAi knockdown of p190 RhoGAP was also shown to block PAR-6-induced RhoA inactivation, suggesting that PAR-6 inhibits RhoA activity through p190 RhoGAP. The mechanism by which PAR-6/aPKC regulates p190 RhoGAP activity remain to be determined, although it is possible that aPKC activates p190 by direct phosphorylation (Brouns *et al.*, 2000). Taken together, these results indicate that PAR-3 and PAR-6/aPKC regulate hippocampal neuron spine morphogenesis by controlling different aspects of Rho GTPase signaling; PAR-3 promotes spine formation by inducing local Rac activation, whereas PAR-6/aPKC controls spine development by suppressing RhoA activity.

In addition to regulating synaptic structure, 190 RhoGAP has been implicated in fear memory formation in the lateral amygdala (LA) through its interaction with a Grb2-mediated molecular complex (Lamprecht *et al.*, 2002). Grb2 is an SH2/SH3 domain-containing adaptor protein that forms multi-protein complexes with other signaling molecules, resulting in the propagation of intracellular signals (Buday, 1999). Following fear conditioning, Grb2 forms a complex with the tyrosine phosphorylated proteins p190 RhoGAP, RasGAP and Shc in the LA (Lamprecht *et al.*, 2002). Since tyrosine phosphorylation of p190 RhoGAP by Src inhibits its GAP activity towards RhoA (Roof *et al.*, 2000), the appearance of tyrosine phosphorylated p190 RhoGAP following fear conditioning suggests that RhoA activation may be involved in fear conditioning. Indeed, inhibition of the Rho/ROCK pathway by microinjection of the ROCK inhibitor Y-27632 into the LA impairs long-term but not short-term fear conditioning (Lamprecht *et al.*, 2002). Given that p190 RhoGAP regulates spine morphogenesis, these data provide a possible link between structural remodeling of synapses mediated by Rho GTPase signaling and long-term memory formation. As is the case for the other GAPs that we have examined, not enough is known about p190 RhoGAP to fully appreciate the nature of its unique role in synaptogenesis, though its many protein-interaction domains and association with Src, Arg and PAR6 provide ample clues.

7. Concluding remarks

As key regulators of cytoskeletal dynamics, Rho GTPases control many different aspects of nervous system development, including neuronal migration, axon growth and guidance, dendrite arborization, synapse formation, and spine morphogenesis (Govek *et al.*, 2005). How do Rho GTPases regulate such diverse cellular processes? Rho GTPases require both precise spatio-temporal regulation of their activities and the activation of particular downstream pathways in order to dynamically control these distinct processes in response to extracellular cues (Pertz, 2010). Rho-family GEFs and GAPs likely provide this signaling specificity. As described in this review, Rho GTPase regulatory proteins typically contain an assortment of functional domains in addition to their GEF or GAP domains. These signaling domains enable Rho GEFs and GAPs to precisely control Rho GTPase activity in space and time and couple it to specific upstream receptors and downstream effector molecules by acting as scaffolding proteins (Moon and Zheng, 2003; Rossman *et al.*, 2005; Schmidt and Hall, 2002). Localized Rho GTPase signaling then induces specific actin and microtubule remodeling that is required for the different neurodevelopmental processes.

In the last ten years, a number of Rho-family GEFs and GAPs have been identified that play critical roles in different aspects of synapse development and plasticity (Kiraly *et al.*, 2010). In general, Rac- and Cdc42-GEFs promote the formation and/or maturation of synapses and spines, whereas Rho-GEFs induce spine shrinkage and synapse elimination. Likewise, Rac-GAPs restrict dendritic protrusions and synapse formation, whereas Rho-GAPs prevent spine/synapse retraction. Since Rho-family GEFs or GAPs with a common GTPase target appear to act in a similar manner at synapses, it is surprising that multiple Rho regulatory proteins have been found to function in the same signaling pathway. For example, the Rac-GEFs Kalirin-7 and Tiam1 have both been shown to regulate synapse development downstream of EphB and NMDA receptors (Penzes *et al.*, 2003; Tolias *et al.*, 2005; Tolias *et al.*, 2007; Xie *et al.*, 2007). Why would more than one Rac-GEF be required to mediate the effects of a particular receptor? It is possible that Kalirin-7 and Tiam1 function at different times during development, in distinct brain regions, and/or in different types of neurons or synapses. Alternatively, since Kalirin-7 and Tiam1 associate with distinct multi-protein complexes via their unique protein-interaction domains, they may have non-overlapping roles in regulating different aspects of Rac signaling at the same synapse. The specific roles of the individual Rho-family regulatory proteins and the mechanistic details of how they act in concert to regulate synapse development is currently unclear, and would benefit from further comparative analysis.

To achieve precise spatio-temporal control of Rho GTPase signaling that is required for proper synapse development and plasticity, the actions of Rho-family GEFs and GAPs likely need to be coordinately regulated. This possibility has been demonstrated by the finding that members of the PAR polarity complex regulate spine morphogenesis by modulating the activities of a Rac-GEF and a Rho-GAP. The polarity protein PAR-3 spatially restricts Tiam1 and induces local Rac activation at spines (Zhang and Macara, 2006), whereas PAR-6 induces p190 RhoGAP-mediated RhoA inhibition (Zhang and Macara, 2008). Since Rac and RhoA have opposite effects on spine morphogenesis, by enhancing Rac activity and suppressing RhoA activity, members of the PAR polarity complex appear to tilt the balance between Rac and RhoA towards Rac-dependent spine formation. It is also possible that Rho-family GEFs and GAPs with specificity for the same Rho GTPase function together to precisely regulate Rho GTPase activity in space and time. Further investigation is required to understand how Rho regulatory proteins act in concert to direct synapse development and plasticity.

In this review, we have focused our attention on Rac-, RhoA- and Cdc42-specific regulatory proteins that play critical roles in synapse formation and remodeling. While regulation of Rac, RhoA and Cdc42 signaling is clearly important for proper synapse development, it is unlikely that these GTPases are the only Rho family members functioning at synapses. Indeed, the novel Rho GTPase Rnd1 has been shown to promote the elongation and maturation of spines by inhibiting RhoA activity (Ishikawa *et al.*, 2003). Currently, more than 20 mammalian Rho-family GTPases have been identified, however little is known about the functions of most of these Rho GTPase family members in neurons. Based on their ability to regulate cytoskeletal dynamics and cell morphology, it will be important to investigate whether any additional Rho GTPases play a role in synapse development and/or plasticity.

Given the essential roles that Rho GTPases play in regulating spine morphogenesis and the strong association between spine abnormalities and mental retardation, it is not surprising that mutations in a number of genes involved in Rho GTPase signaling have been found to cause mental retardation in humans (Govek *et al.*, 2005; Ramakers, 2002). These genes include Rho GTPase GEFs (ARHGEF6, FGD1), GAPs (OPHN1, OCRL1, MEGAP) and downstream signaling molecules (PAK3, FMR1) (Allen *et al.*, 1998; Attree *et al.*, 1992; Billuart *et al.*, 1998; Billuart and Chelly, 2003; Endris *et al.*, 2002; Kutsche *et al.*, 2000; Lebel *et al.*, 2002; Soderling *et al.*, 2003). The connection between altered Rho GTPase signaling, spine abnormalities, and mental retardation suggests that proper Rho GTPase signaling is important for normal cognitive development. *In vivo* studies in mice lacking Rho GTPases and their regulatory proteins will help to clarify the individual roles these proteins play in synapse development and plasticity and provide insight into how disruptions in Rho GTPase signaling could give rise to cognitive disorders such as mental retardation.

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Abbreviation List

GEF	Guanine nucleotide exchange factor
GAP	GTPase-activating protein
LTP	Long-term potentiation
LTD	Long-term depression
F-actin	filamentous actin
CNS	central nervous system
PH	pleckstrin homology domain
CC-Ex	coiled coil-extended region
RBD	Ras-binding domain
PDZ	PSD-95, Dlg, and ZO-1/2 domain
DH	Dbl homology domain
SEC14	Sec14p homology domain
SPEC	spectrin-like repeats
SH3	Src homology 3 domain

EH	Eps15 homology domain
C1	protein kinase C conserved region 1
C2	protein kinase C conserved region 2
BAR	Bin/Amphiphysin/Rvs domain
P	proline-rich regions
PSD	post-synaptic density
NMDA receptor	N-methyl-D-aspartate receptor
AMPA receptor	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
PAR	partition-defective protein
aPKCζ	atypical protein kinase C
Pak	p21-activated kinase
CaMKK	calmodulin-dependent kinase kinase
mEPSC	miniature excitatory postsynaptic current
N-WASP	neuronal Wiskott-Aldrich syndrome protein
Cdk5	cyclin dependent kinase 5
ASDs	Autism Spectrum Disorders
NMJ	neuromuscular junction
GABA receptor	gamma-amino butyric acid receptor
Ach receptor	acetylcholine receptor
DAG	diacyglyerol
Bcr	breakpoint cluster region
Abr	active Bcr-related
CIN85	c-Cbl-interacting protein of 85 kDa
Arg	Abl-related gene
LA	lateral amygdala
Grb2	growth factor receptor-bound protein 2
ROCK	Rho-associated coil-containing protein kinase

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Research Highlights

- Proper synapse development and plasticity are essential for normal cognitive function.
- Rho GTPases, key regulators of the actin cytoskeleton, control the formation and structural remodeling of synapses.
- Precise spatio-temporal regulation of Rho GTPase signaling is critical for their function.
- Rho GTPase regulatory proteins direct synapse development and plasticity by controlling the spatio-temporal regulation and signaling specificity of Rho GTPases.

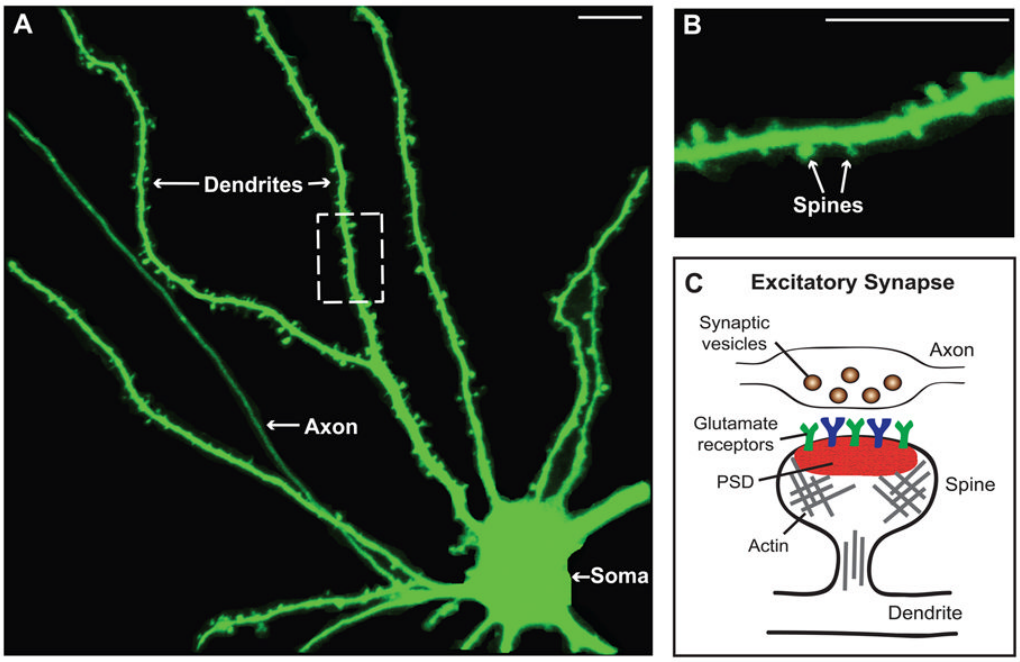

Figure 1. Dendritic spines are the primary sites of excitatory synapses in the brain

A. Shown is an example of a rat hippocampal neuron expressing green fluorescent protein (GFP). Neurons possess a soma, an axon and branched dendrites containing dendritic spines. B. Image shows an enlargement of the dashed box pictured in A, which provides a clearer view of spines. C. Schematic of an excitatory synapse, which forms between a dendritic spine and a presynaptic bouton on an axon. The postsynaptic density (PSD), which contains glutamate receptors, scaffolding proteins and other signaling molecules, is located on the spine head. Spines are also enriched in actin filaments. Scale bar: 10 νm

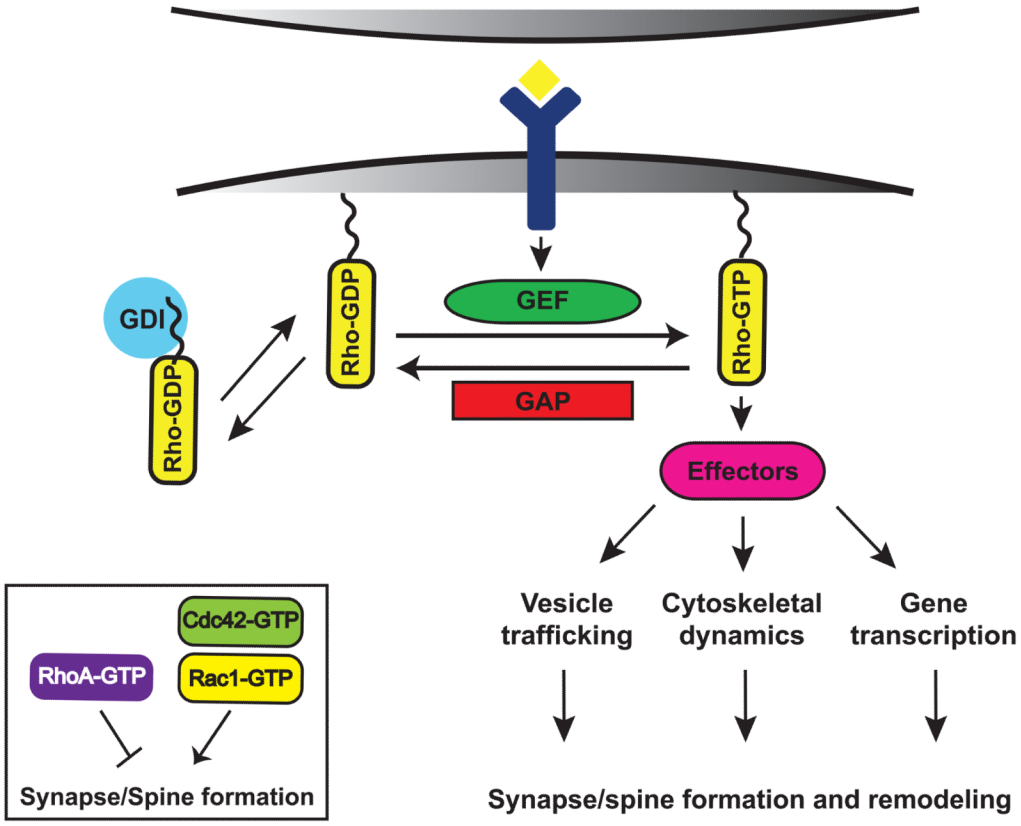

Figure 2. Model of Rho GTPase signaling at synapses

Rho GTPases function as binary switches by cycling between an active, GTP-bound form and an inactive, GDP-bound form. Rho GTPase activity is tightly regulated in space and time by three different classes of regulatory proteins: guanine nucleotide exchange factors (GEFs), GTP-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). In their active state, Rho GTPases interact with downstream effectors that regulate a variety of cellular processes, which ultimately contribute to spine morphogenesis and excitatory synapse development. The Rho GTPases Rac and Cdc42 promote the formation and growth of synapses and spines, whereas RhoA inhibits synapse development.

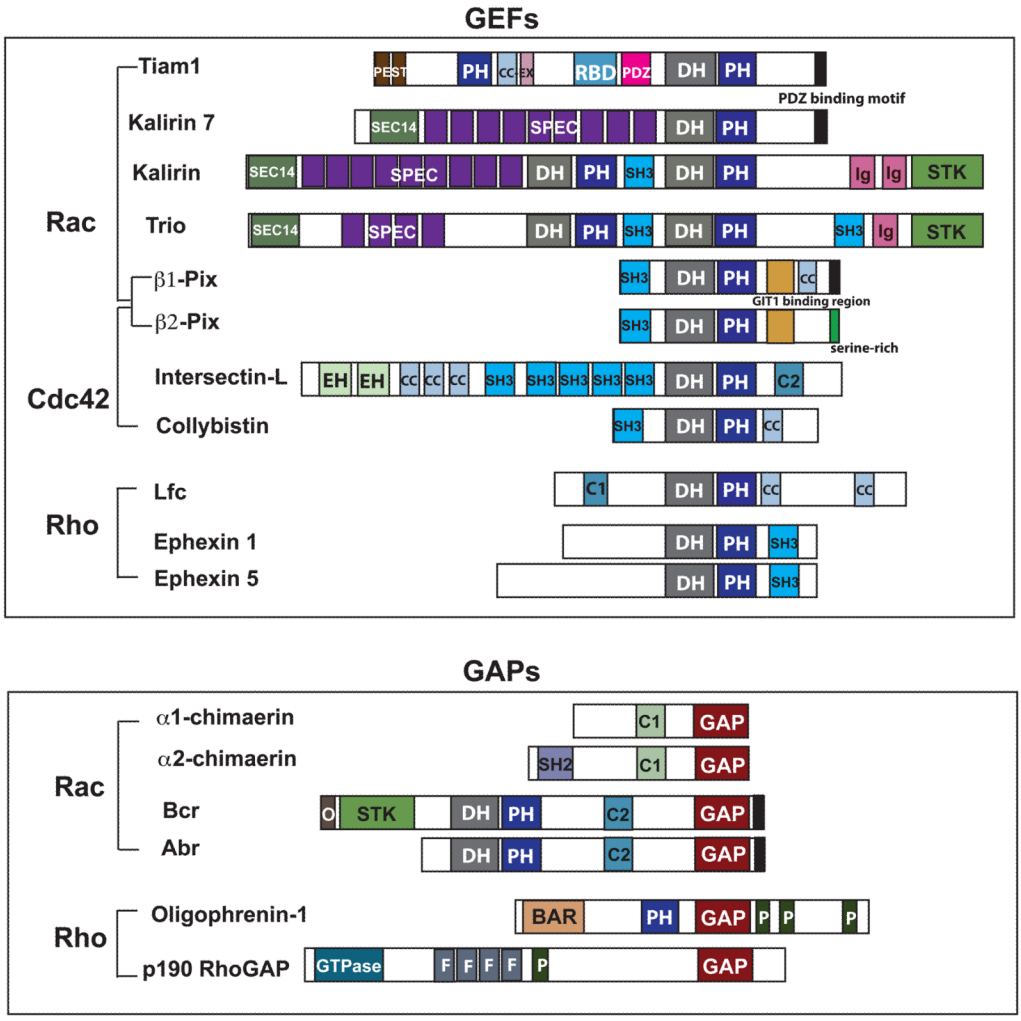

Figure 3. Domain structures of synaptic Rho-family GEFs and GAPs

Shown are the domain structures of proteins mentioned in this review. The following abbreviations are used in this figure: **PH:** pleckstrin homology domain, **CC-Ex:** coiled coil-extended region, **RBD:** Ras-binding domain, **PDZ:** domain in PSD-95, Dlg, and ZO-1/2, **DH:** Dbl homology domain, **SEC14:** domain in phosphatidylinositol transfer protein Sec14, **SPEC:** spectrin-like repeats, **SH3:** Src homology 3 domain, **EH:** Eps15 homology domain, **C2:** protein kinase C conserved region 2, **C1:** protein kinase C conserved region 1, **BAR:** Bin/Amphiphysin/Rvs domain, and **P:** proline-rich regions.

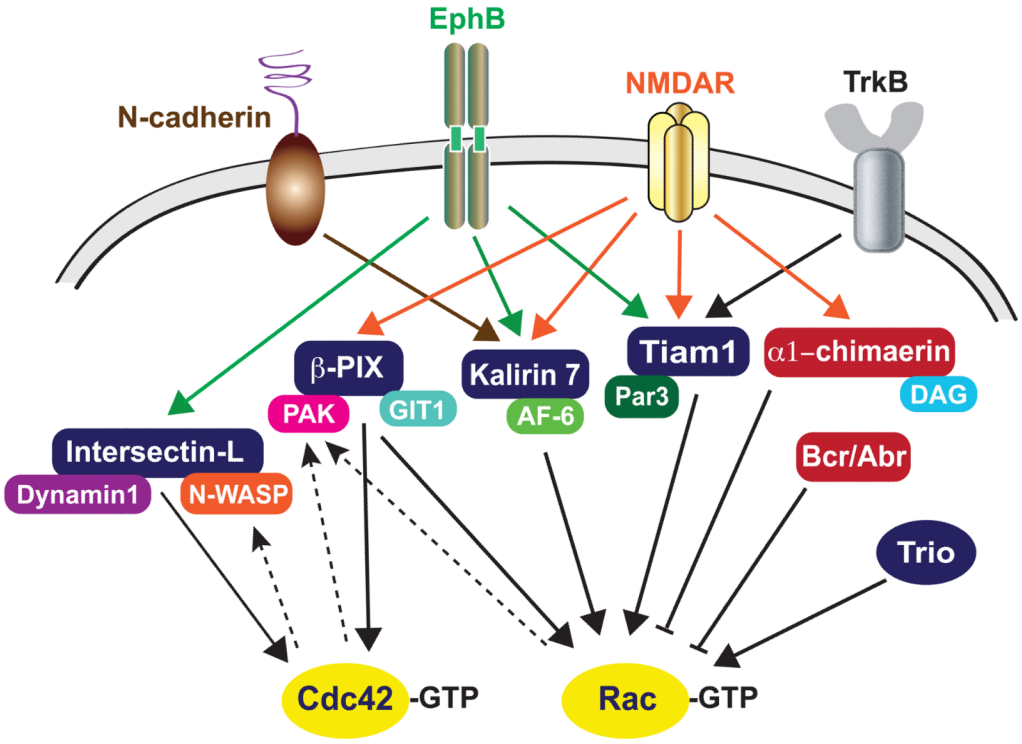

Figure 4. Regulation of Rac/Cdc42 signaling at synapses

The Rac/Cdc42-GEFs Tiam1, Kalirin7, β-PIX, Intersectin-L and Trio promote Rac/Cdc42 activation, whereas the Rac-GAPs α1-chimaerin, Bcr and Abr inhibit Rac activation at synapses. Although these GEFs and GAPs are regulated by common upstream receptors, e.g. NMDA receptors, EphB, TrkB and N-cadherins, the unique role of each regulator likely arises from the networks of interacting proteins that are unique to each GEF or GAP and/or differential upstream regulation of these molecules in a temporal and spatial manner. GEFs are in blue and GAPs are in red, and dashed lines represent positive feedback loops.

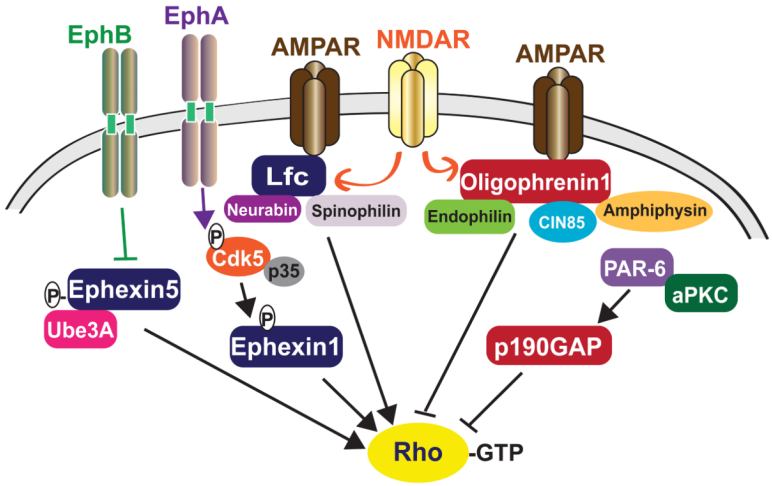

Figure 5. Regulation of RhoA signaling at synapses

The RhoA-GEFs Lfc, Ephexin1 and Ephexin5 activate RhoA activity, whereas the RhoA-GAPs Oligophrenin1 and p190 RhoGAP inhibit RhoA activity. These regulatory proteins are controlled by upstream receptors, such as NMDA receptors, AMPA receptors, and Eph receptors. As is the case for Rac1/Cdc42 GEFs and GAPs, RhoA regulatory proteins likely achieve their specific functions through a unique set of interactions with downstream effectors and other proteins. GEFs are in blue and GAPs are in red.