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Diverse roles of Rho family GTPases in neuronal development, survival, and death

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1. ABSTRACT

Rho family GTPases (*eg.*, RhoA, Rac1 and Cdc42) are monomeric G-proteins that act as key transducers of extracellular signals to the actin cytoskeleton. In the nervous system, Rho family GTPases are essential regulators of neuronal growth cone motility, axonal migration, and dendritic spine morphogenesis. Given these vital functions, it is perhaps not surprising that mutations in several proteins involved in Rho GTPase signaling are causative in some forms of mental retardation. In addition, numerous recent studies have identified Rho family GTPases as central players in the molecular pathways that determine neuronal survival and death. Interestingly, individual Rho family members have been shown to play either a pro-death or pro-survival role in the nervous system depending on both the type of neuron and the particular neurodegenerative insult involved. This review summarizes current work demonstrating a critical role for Rho family GTPases and their effectors in the regulation of neuronal development, survival, and death. These findings may be particularly relevant in the context of specific neurodegenerative disorders in which Rho family GTPase function is altered, such as loss-of-function of the Rac1 guanine nucleotide exchange factor, alsin, in juvenile-onset amyotrophic lateral sclerosis.

2. INTRODUCTION

Rho family GTPases are members of the Ras superfamily of low molecular weight, monomeric G-proteins (1). The Rho family has recently been divided into six GTPase subfamilies including Rho, Rac, Cdc42, Rnd, RhoBTB and RhoT/Miro (2). The most well studied members of this family include the RhoA, Rac1 and Cdc42 proteins which were originally identified as key regulators of actin cytoskeletal dynamics. Specifically, RhoA induces formation of actin stress fibers and focal adhesions, Rac1 stimulates protrusion of lamellipodia and membrane ruffles, and Cdc42 promotes extension of filopodia and actin microspikes (3-5). In addition to their prominent effects on the actin cytoskeleton, Rho family GTPases also modulate other important cell functions including membrane trafficking, cell cycle progression, gene transcription, adhesion, migration, and survival (6-11). Rho GTPases are converted from an inactive (*GDP-bound*) state to an active (*GTP-bound*) form downstream of ligand-bound integrins, growth factor receptors and heterotrimeric G-protein-coupled receptors (GPCRs). The activation of Rho GTPases is regulated by two major classes of proteins: guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GTP for GDP and GTPase-activating proteins (GAPs) which stimulate the intrinsic GTPase

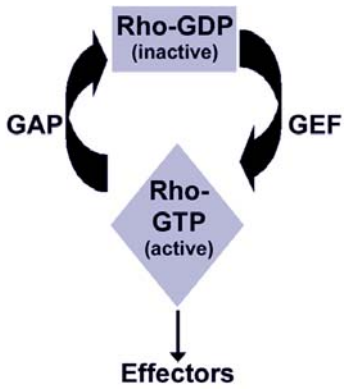

Figure 1. Cycle of Rho GTPase activation. Enabled by GEFs which stimulate the exchange of GTP for GDP, inactive Rho-GDP undergoes a conformational change to active Rho-GTP that binds and stimulates downstream effector proteins. GAPs then stimulate the intrinsic GTPase activity of Rho to hydrolyze GTP to GDP, returning it to an inactive conformation. GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor.

activity of Rho family members to hydrolyze GTP to GDP (12, 13) (Figure 1). The binding of GTP induces a conformational change in Rho GTPases which promotes their association with a diverse array of downstream effectors including protein kinases, lipid kinases, scaffolding proteins, phospholipases and others (reviewed in 2). This large pool of effector proteins, as well as the multitude of GEFs and GAPs known to modulate the activity of Rho GTPases, creates a complex signaling network.

In the nervous system, Rho family GTPases and their downstream effectors play major roles in the development, migration, and plasticity of neurons. In *Drosophila* for example, Rac GTPases control axonal growth, guidance and branching in both mushroom body neurons and motor neurons (14, 15). Moreover, *Drosophila* mutants which are deficient in activity of the Rac GEF, Trio, show deficits in axon guidance that are analogous to those observed in Rac mutants (16). In a similar manner, Rac GTPases also modulate axon pathfinding and neuronal migration in *C. elegans* (17). In the vertebrate embryo, changes in the expression levels of numerous members of the Rho GTPase family correlate with development of the nervous system (18). Rho, Rac and Cdc42 have all been shown to regulate dendritic growth and arborization, as well as axon guidance (19-21). In particular, Rac activation appears to play a key role in vertebrate nervous system development. For example, the Rac GEF, Tiam1, is highly expressed in migrating cerebellar granule neurons (CGNs) of wild-type mice, but not in CGNs of weaver mutant mice which exhibit deficits in CGN migration (22). Moreover, Tiam1 has been shown to enhance neurite outgrowth and axon formation via its activation of Rac (23, 24). In addition to Tiam1, several other Rac activators have been implicated in neuronal development including STEF, P-Rex1 and DOCK7 (25-28). Finally, conditional deletion of Rac1 in the ventral telencephalon of mice prevents axonal migration across the midline of the corpus callosal and hippocampal commissures, further demonstrating a critical role for Rac1 in axon guidance during development of the vertebrate central nervous system (CNS) (29).

Given the critical roles of Rho family GTPases in CNS development, one might accurately predict that compromising the signal transduction networks that are normally regulated by these G-proteins would adversely affect specific brain functions such as cognition (30). A somewhat less obvious prediction regarding the potential functions of Rho family GTPases in the nervous system is the recent observation that these G-proteins are essential regulators of neuronal survival. In the present review, we will briefly summarize the well known roles of Rho family GTPases in the regulation of neuronal growth cone dynamics and dendritic spine morphogenesis, with an emphasis on the relationship of these functions to cognitive development. We will then highlight the current literature indicating that Rho family GTPases can also play either pro-death or pro-survival roles in neurons in a context-dependent manner. Finally, we will examine the potential clinical relevance of the recently recognized function of Rho family GTPases to critically influence neuronal survival.

3. RHO GTPASE EFFECTORS

Prior to discussing specific aspects of Rho GTPase function, we will first give a brief overview of Rho, Rac and Cdc42 signaling to downstream effector protein kinases. The reader is directed to several previous reports for a more comprehensive review of Rho GTPase effectors (2, 31, 32).

3.1. Rac/Cdc42/PAK

Rho family GTPases transduce signals from the extracellular environment following the activation of various integrins, growth factor receptors, and GPCRs. Once in proximity to an activated receptor at the plasma membrane, Rho GTPases are subsequently targeted by GEFs. After a specific GEF has catalyzed the exchange of GTP for GDP, the Rho GTPase changes to an active conformation that allows it to associate with and stimulate specific downstream effector molecules (Figure 1). In the case of Rac and Cdc42, effector proteins such as the p21-activated kinases (PAKs) interact with these GTPases via a Cdc42/Rac-interactive binding (CRIB) motif (33). The PAKs are a family of serine/threonine protein kinases that can be activated downstream of either Rac or Cdc42 GTPases. The mammalian PAK family consists of six members (PAK 1-6) which are subcategorized into two groups (A and B) (34, 35). Group A PAKs (PAK 1-3) are characterized by a proline-rich region that facilitates their interactions with Src homology-3 (SH3) domain proteins. In addition, the kinase activity of group A PAKs is significantly enhanced by their association with Rac/Cdc42 GTPases. In contrast, group B PAKs (PAK 4-6) do not show a marked increase in kinase activity upon binding Rac/Cdc42 GTPases; instead, this binding appears to act as

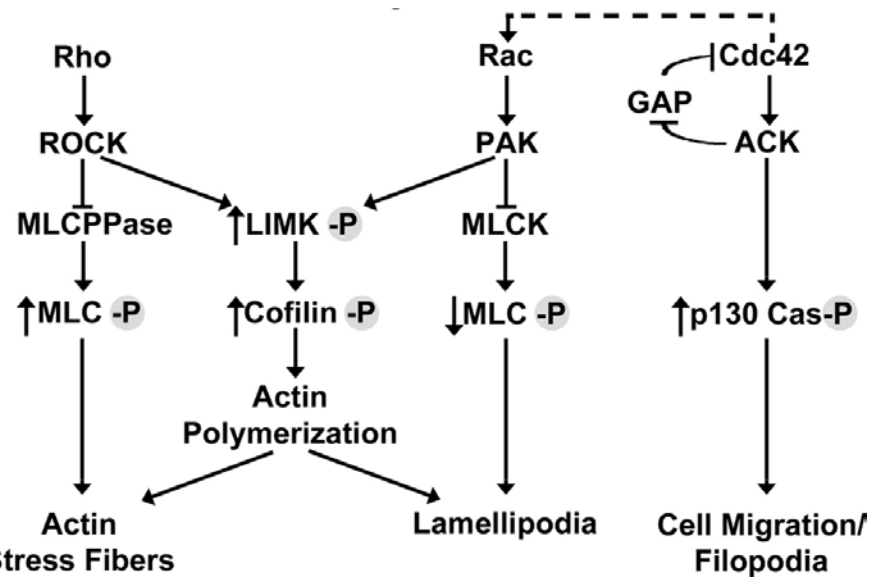

Figure 2. Rho GTPase signaling to downstream effector protein kinases involved in cytoskeletal remodeling. Rho signals through ROCK to increase MLC phosphorylation and cofilin phosphorylation, leading to formation of actin stress fibers. In contrast, Rac signals through PAK to decrease MLC phosphorylation while increasing cofilin phosphorylation, thereby inducing lamellipodia. Cdc42 stimulates the tyrosine kinase, ACK, resulting in the phosphorylation of p130Cas, enhanced cell migration, and increased filopodial extensions. Under some conditions, Cdc42 can also act upstream of Rac-dependent signaling (dotted line). ACK, activated Cdc42-associated kinase; Cas, Crk-associated substrate; GAP, GTPase-activating protein; LIMK, LIM kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCPPase, myosin light chain phosphatase; PAK, p21-activated kinase; ROCK, Rho kinase.

a mechanism to localize active PAK molecules to the site of GTPase action.

The activation of PAK downstream of GTP-bound Rac/Cdc42 induces several key signaling cascades (Figure 2). First, PAK has been shown to directly phosphorylate myosin light chain kinase (MLCK) which diminishes MLCK activity and consequently reduces phosphorylation of the myosin II regulatory light chain (36, 37). Myosin II is a key molecular motor protein involved in the organization of the actin cytoskeleton, and its ability to modulate cytoskeletal dynamics is significantly regulated by phosphorylation (38). Another major cytoskeleton-associated target of PAK is LIM kinase; phosphorylation by PAK stimulates LIM kinase-mediated phosphorylation of the actin capping protein, cofilin (39). Phosphorylation of cofilin inhibits its ability to depolymerize F-actin, resulting in the stimulation of actin reorganization which is required for Rac-dependent formation of lamellipodia and membrane ruffles (40). In addition to the cytoskeleton-associated substrates, MLCK and LIM kinase, PAK is also an upstream activator of several mitogen-activated protein kinase (MAPK) pathways. For example, PAK directly phosphorylates both Raf1 and MEK1 leading to activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (41, 42). Moreover, PAK has also been shown to link Rac/Cdc42 signaling to the c-Jun-NH_2-terminal kinase (JNK) and p38 MAPK pathways (43, 44). Finally, in the context of cell survival, PAK itself is directly phosphorylated by AKT and in turn, both AKT and PAK phosphorylate the pro-apoptotic Bcl-2 family member, Bad, which prevents its mitochondrial localization and death promoting activity (45-47). Thus, PAK is a critical downstream effector of Rac/Cdc42 GTPase signaling which has many crucial functions in neurons (discussed below).

3.2. Rho/ROCK

Like Rac/Cdc42 signaling to PAK, Rho also activates a key downstream kinase, the Rho-associated coiled-coil forming protein serine/threonine kinase, ROCK (48-50) (Figure 2). Currently, there are two known isoforms of ROCK in mammals, ROCK1 and ROCK2, with ROCK2 showing relatively higher levels of expression in brain (49). Under many conditions, these two ROCK isoforms appear to act in a redundant manner downstream of Rho GTPase. In some circumstances, the Rho/ROCK pathway directly opposes Rac/PAK signaling. For example, while stimulation of PAK reduces the phosphorylation of myosin light chain (36, 37), ROCK directly phosphorylates and inhibits the activity of myosin phosphatase which has the net effect of enhancing the phosphorylation of myosin light chain (51). On the other hand, in the case of LIM kinase, ROCK acts in a manner analogous to PAK and phosphorylates LIM kinase at a common site (Thr-508) resulting in an increase in its activity (52, 53). Consequently, both Rho/ROCK and Rac/PAK induce the LIM kinase-dependent phosphorylation of cofilin and enhance actin polymerization, a prerequisite for the ability of each of these GTPase pathways to ultimately reorganize the actin cytoskeleton. Yet another example of congruent signaling

between Rho/ROCK and Rac/PAK is the capacity of Rho to activate the JNK pathway through a ROCK-dependent mechanism (54). Although Rho/ROCK and Rac/PAK are commonly regarded as antagonistic pathways, this is not always evident at the molecular level when their effects on specific downstream signaling proteins (*eg.*, LIM kinase and JNK) are examined. Like PAK, ROCK plays an essential role in neuronal physiology (discussed below).

3.3. Cdc42/ACK

In addition to serine/threonine protein kinases like PAK and ROCK, specific tyrosine kinases are also downstream effectors of Rho family GTPases. In particular, the activated Cdc42-associated kinase (ACK) is a non-receptor tyrosine kinase that specifically interacts with GTP-bound Cdc42 (55) (Figure 2). The mammalian ACK family consists of at least three known members, ACK-1, ACK-2 and Tnk (55-57). The association of ACK with GTP-bound Cdc42 not only enhances the tyrosine kinase activity of ACK, but it also has an inhibitory effect on both the intrinsic and GAP-stimulated GTPase activity of Cdc42 (55). This effect of ACK is unique among many Rho GTPase effector proteins in that the binding of ACK to Cdc42 actually induces a prolonged active (GTP-bound) state of the GTPase. ACK tyrosine kinase activity is stimulated in a Cdc42-dependent manner downstream of integrins, growth factor receptors and GPCRs (56, 58, 59). The Cdc42/ACK pathway is associated with activation of the Rho GEF, Dbl, and consequent downstream stimulation of JNK (60). ACK also associates with clathrin-coated vesicles and ubiquitin, and contributes to growth factor receptor internalization and degradation (61, 62). Finally, ACK plays a key role in Cdc42-dependent cell migration by phosphorylating the adapter protein, p130Cas, a critical component of migratory signaling complexes (63). In contrast to PAK and ROCK, the precise role of ACK signaling in neurons is not well defined.

4. RHO GTPASES, NEURONAL GROWTH CONE DYNAMICS, AND DENDRITIC SPINE MORPHOGENESIS

Because Rho family GTPases are prominent regulators of the actin cytoskeleton, it is predictable that they play major roles in neurons via modulation of growth cone morphology. In an elegant series of experiments utilizing microinjection of wild-type and dominant-negative mutant GTPases, Rho inhibitory Clostridial toxins, and specific GPCR agonists in N1E-115 neuroblastoma cells, Kozma *et al.* (1997) showed that Rac and Cdc42 promote growth cone advance and neurite outgrowth while Rho causes growth cone collapse and neurite retraction (64). A similar antagonistic relationship between Rac/Cdc42 and Rho was reported for axonal pathfinding of neurons responding to the guidance molecule netrin-1. Both Rac and Cdc42 activities are required for axon outgrowth induced by netrin-1, and this effect is mimicked by downregulation of Rho and ROCK (65). Analysis of the effector proteins involved in neuronal growth cone remodeling downstream of Rho GTPases has revealed an essential role for PAK in neurite outgrowth induced by Rac/Cdc42 (66). For example, dominant-negative PAK mutants inhibit neurite outgrowth of PC12 cells stimulated by nerve growth factor (NGF), and membrane targeting of active PAK (via an isoprenylation motif) is sufficient to induce neurite outgrowth (67). Moreover, PAK functions as part of a protein complex containing Rac GTPase and the adapter molecule, DOCK, to promote axon pathfinding in both *Drosophila* photoreceptors and olfactory neurons (68-70). In general, Cdc42 appears to cooperate with Rac to stimulate neurite outgrowth, although the precise morphology of growth cones responding to these distinct GTPases display some visible differences (71, 72). Additionally, under some conditions like NGF-stimulated neurite outgrowth, Cdc42 and Rac appear to act in a sequential manner to stimulate neuritogenesis (73). In opposition to Rac/Cdc42 signaling, Rho induces neurite retraction and growth cone collapse through several mechanisms, some of which require the protein kinase ROCK. For instance, in both ephrin A5- and semaphorin 3A-induced growth cone collapse and axon retraction, inhibition of ROCK activity prevents growth cone remodeling (74, 75). Finally, it is noteworthy that under specific conditions, the role of Rac on growth cone morphology is apparently altered to work in conjunction with Rho. Specifically, growth cone collapse induced by semaphorin 3A or 3D has been shown to occur via Rac activation as well as through Rho/ROCK signaling (76, 77). Intriguingly, this occurs through a convergence of Rac and Rho signals where ROCK phosphorylates the collapsin response mediator protein, Crmp-2, which in turn alters the activity of Rac to induce the growth cone to collapse rather than advance (78). The above findings demonstrate the intricate balance of Rho GTPase signals that underlies the dynamic regulation of growth cone remodeling and axon pathfinding which is required for proper nervous system development and function.

In addition to the aforementioned effects on growth cone dynamics, Rho family GTPases are also dominant regulators of dendritic spine morphogenesis and therefore, they play key roles in synapse formation and nervous system plasticity. Just as Rac and Rho typically antagonize one another in their effects on growth cone motility and axon pathfinding, these two GTPases also have opposing effects on dendritic spine morphology. In general, Rac promotes and Rho diminishes dendritic spine formation (79-81). These Rho GTPases modulate dendritic spine morphology and synaptogenesis downstream of integrin- and cadherin-mediated adhesion processes (82, 83). Rac regulates spine morphogenesis following the activation of a diverse group of GEFs including alpha-p21-activated kinase interacting exchange factor (alphaPIX), also known as Cool-2 or ARHGEF6, and Tiam1 (84-86). In the case of alphaPIX/ARHGEF6, PAK1 and/or PAK3 have been shown to be key downstream effectors of Rac required for dendritic spine formation (84, 86). Conversely, stimulation of the Rac GAP, alpha1-chimaerin, suppresses Rac activity and causes a reduction in dendritic spine density (87). In contrast to the well characterized role for Rac/PAK signaling in spine morphogenesis, little is known about the role of Cdc42 in this process. One recent study showed that the specific Cdc42 effector, ACK-1, is highly expressed in dendritic spines of neurons in the

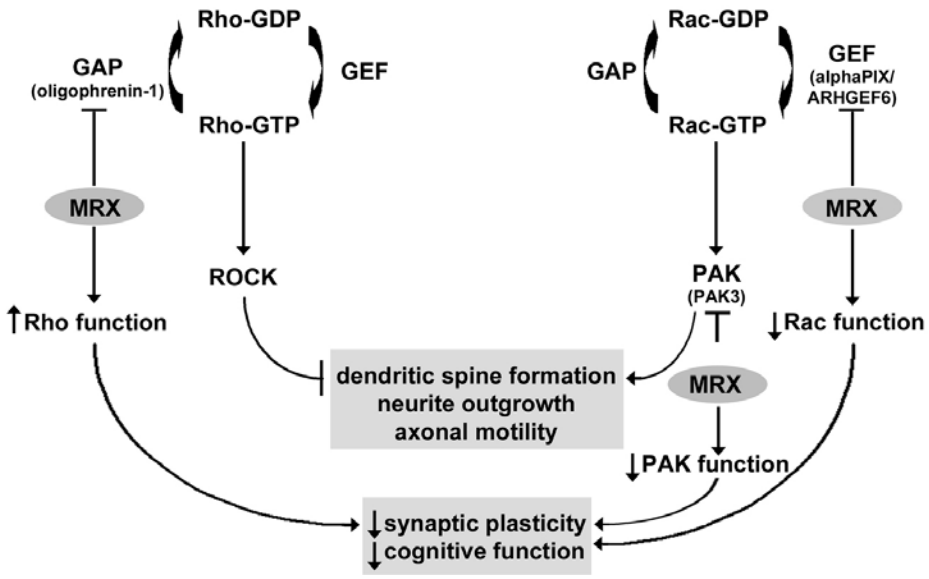

Figure 3. Rho GTPase signaling molecules mutated in nonspecific X-linked mental retardation (MRX). Mutation of the Rho GAP, oligophrenin-1, increases Rho function causing neurite retraction. Mutation of the Rac effector, PAK3, disrupts neurite outgrowth and dendritic spine formation. Finally, mutation of the Rac GEF, alphaPIX/ARHGEF6, results in decreased Rac function. Mutations in these three signaling molecules ultimately contribute to decreased synaptic plasticity and deficits in cognitive function. GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; PAK, p21-activated kinase; ROCK, Rho kinase.

hippocampus, cortex and cerebellum, and moreover, ACK-1 mRNA levels are markedly upregulated by kainate-stimulated neural activity (88). These findings suggest a likely role for Cdc42/ACK signaling in synaptic plasticity. Lastly, the ability of Rho to antagonize Rac/PAK signaling and dendritic spine formation is predictably dependent on the activity of ROCK (79, 81).

The critical role of Rho GTPases in synaptic plasticity is further demonstrated by an examination of the signaling pathways that underlie the effects of N-methyl-D-aspartate (NMDA) receptors and ephrin/Eph receptors on dendritic spine architecture (89). NMDA receptor activation typically enhances dendritic spine formation, and two recent studies showed that NMDA receptors also modulate the activities of diverse Rho GAPs. For example, the NR2B subunit of the NMDA receptor interacts with p250GAP, a GAP capable of inactivating Rho but not Rac in dendritic spines (90). Another GAP that shows selectivity for Rac and Cdc42, RICS, is also associated with NMDA receptors and its activity is inhibited by calcium-dependent phosphorylation (91). Thus, NMDA receptor activation is predicted to enhance activity of the p250GAP leading to the inactivation of Rho in dendritic spines, while concurrently suppressing RICS via calcium-dependent phosphorylation and consequently enhancing Rac function. The net effect of decreased Rho-GTP and increased Rac-GTP would be stimulation of dendritic spine formation following NMDA receptor activation. Similarly, diverse members of the ephrin/Eph receptor family induce either spine formation or retraction via mechanisms that are dependent on Rho GTPase signaling. For instance, stimulation of the ephrinB/EphB ligand/receptor complex in hippocampal neurons induces the translocation of a Rac GEF, kalirin, to synapses and subsequently activates a Rac/PAK signaling pathway which is required for dendritic spine morphogenesis (92). On the other hand, stimulation of the EphA4 receptor triggers retraction of dendritic spines through a mechanism that involves the activation of a specific Rho GEF, ephexin1, and consequent GTP-loading of Rho (93). The literature cited above clearly demonstrates that Rho family GTPases are essential regulators of dendritic spine morphology and synapse formation. Thus, it is evident that disruption of Rho GTPase signaling pathways is undoubtedly involved in CNS disorders characterized by diminished or aberrant synaptic plasticity such as cognitive impairment.

5. RHO GTPASES AND COGNITIVE IMPAIRMENT

5.1. Nonspecific X-linked mental retardation

Normal cognitive function is dependent on proper neuronal differentiation and migration, correct axonal pathfinding, and appropriate formation of synapses at contact sites on dendritic spines. Therefore, Rho family GTPase signaling pathways which regulate each of these processes are essential for normal cognitive development. There have been several detailed reviews published on the role of Rho family GTPase signaling in cognitive function and dysfunction (94-96). Interest in this topic has peaked with the recent discoveries that several genes mutated in nonspecific X-linked mental retardation (MRX) are components of Rho GTPase signaling cascades (Figure 3). One such protein is the Rac/Cdc42 effector, serine/threonine kinase PAK3, which is highly expressed in CNS neurons (97-99). To date, three specific mutations

have been identified in PAK3 that each correspond to particular families displaying MRX of varying degrees of severity. The first of these mutations discovered was R419X which causes premature termination of the PAK3 protein resulting in a complete loss of kinase activity (97). The second mutation identified was a missense (R67C) which, given its proximity to the CRIB domain, likely disrupts the interaction of PAK3 with activated Rac/Cdc42 GTPases (98). The third mutation characterized was an A365E substitution within a highly conserved region of PAK3 that is predicted to distort protein conformation and likely disrupt kinase activity (99). Given the critical involvement of PAK proteins in promoting neurite outgrowth, axonal motility, and dendritic spine formation, disruption of PAK signaling would be anticipated to have deleterious consequences to CNS development (66-70, 84, 86, 96). Consistent with loss-of-function of PAK3 playing a causative role in cognitive impairment, PAK3 knock-out mice display abnormal hippocampal synaptic plasticity and corresponding deficits in learning and memory (100).

In addition to downstream effectors like PAK3, other regulators of Rho family GTPases (*ie.*, GEFs and GAPs) are also mutated in MRX. For example, the Rac GEF, alphaPIX/ARHGEF6, is functionally disrupted in MRX (101). Given that alphaPIX/ARHGEF6 is a known activator of Rac-dependent signaling to PAK1/PAK3 and consequent induction of dendritic spine formation, loss-of-function of this GEF is postulated to compromise normal synapse formation (84, 86). In addition, the Rho GAP, oligophrenin-1, is similarly mutated in MRX resulting in its downregulation (102). In cultured rat hippocampal slices, the forced downregulation of oligophrenin-1 by RNA interference causes a marked reduction in the length of CA1 neuron dendritic spines (103). The latter effect is mimicked by expression of constitutively-active RhoA and is prevented by inhibition of ROCK, indicating that normal expression levels of oligophrenin-1 provide an essential function in dendritic spine formation by suppressing Rho/ROCK signaling (103). The above studies demonstrate that genetic disruption of Rho family GTPase signaling pathways is a common underlying cause of MRX.

5.2. Fragile X mental retardation syndrome

The fragile X mental retardation syndrome is a relatively common inherited disorder of cognitive impairment. It is caused by mutation of the FMR1 gene and consequent depletion of the fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that regulates the transport and translation of various mRNA transcripts (104). Several recent studies have shown that FMRP modulates local dendritic protein translation at synapses and in turn, influences synapse number on dendritic spines (105, 106). The potential of FMRP to affect synapse formation is in agreement with its depletion leading to cognitive impairment. Although the molecular mechanisms that control FMRP-dependent protein translation at synapses are unclear, there is some evidence that crosstalk with a Rac GTPase signaling pathway is involved. For example, the ability of FMRP to regulate protein translation in the *Drosophila* nervous system is antagonized by the FMRP interacting protein and Rac effector, CYFIP (107). Conversely, FMRP interferes with some aspects of Rac-dependent actin cytoskeleton restructuring (108). These data suggest that an equilibrium between FMRP-dependent protein translation and Rac-dependent cytoskeletal remodeling may contribute to the formation of functional synapses and neuronal connectivity.

5.3. Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is an autosomal dominant inherited disease characterized by multi-system formation of hamartomas (benign tumor-like nodules) and associated neurological disorders including mental retardation, autism, and seizures (109). Mutations of two related tumor suppressor genes, TSC1 and TSC2, lead to inactivation of their tumor suppressor function and are the underlying causes of this disease. The TSC1 gene product, hamartin, has been shown to activate Rho GTPase through an interaction with the ezrin-radixin-moesin (ERM) family of actin-associated proteins (110). In addition, hamartin apparently inhibits Rac activity through an unidentified mechanism (111). The opposing effects of hamartin on Rho and Rac activity are consistent with the induction of neurite outgrowth observed in NGF-differentiated PC12 cells after antisense-mediated downregulation of hamartin (112). In a similar manner, the TSC2 gene product, tuberin, also activates Rho (113). However, tuberin can additionally activate Rac by antagonizing the inhibitory action of hamartin on this GTPase and therefore, tuberin downregulation inhibits neurite outgrowth in PC12 cells (111, 112). These data indicate that mutations in hamartin and tuberin likely contribute to neurologic dysfunction in TSC by altering Rho family GTPase signaling and consequently affecting the normal pattern of neurite outgrowth in the CNS.

5.4. Down syndrome

Down syndrome (DS) is a prevalent form of mental retardation that is caused by an extra copy of chromosome 21 (trisomy 21) (114, 115). Multiple studies spanning the past thirty years have described prominent abnormalities in dendritic spines and synaptic structures in patients with DS and in the Ts65Dn mouse model of the disease (116-119). Given these observations, it is logical to hypothesize that alterations in Rho family GTPase function may play a role in DS. More than a decade ago, the gene for the Rac-specific GEF, Tiam1, was localized to chromosome 21 suggesting a potential role for aberrant expression of this Rac activator in DS (120). Although one subsequent report found increased levels of Tiam1 in the bone marrow of DS children suffering from associated acute myeloid leukemia, Tiam1 expression in fetal DS brain appears to not be increased above normal levels (121, 122). Thus, the role of Tiam1 in DS remains controversial. Another protein of interest in DS that has recently been linked to Rho GTPase signaling is the tetratricopeptide repeat domain 3 (TTC3) protein. The gene that encodes the TTC3 protein is found within the DS Critical Chromosomal Region (DSCR) on chromosome 21, and mice polytransgenic for several of the genes in this region, including TTC3, demonstrate cognitive deficits and alterations in hippocampal proteins that resemble changes

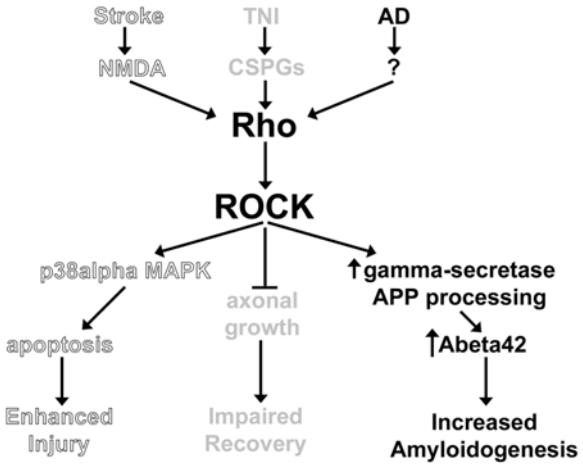

Figure 4. Rho/ROCK signaling plays a central role in neuronal injury and disease. First, ischemia or excitotoxicity activates Rho and its downstream effector, ROCK, inducing a p38alpha MAPK-dependent pro-apoptotic signaling cascade that enhances neuronal injury. Second, recovery after TNI is significantly impaired due to CSPG-induced Rho/ROCK signaling and consequent inhibition of axonal growth. Finally, Rho/ROCK signaling promotes gamma-secretase-dependent APP processing and subsequent increased production of Abeta42, which contributes to amyloidogenesis in AD. AD, Alzheimer's disease; APP, amyloid precursor protein; CSPGs, chondroitin sulfate proteoglycans; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; ROCK, Rho kinase; TNI, traumatic nerve injury.

observed in DS (123, 124). Recently, TTC3 has been shown to interact with the Rho GTPase effector, citron kinase (CITK), as part of a TTC3/RhoA/CITK signaling pathway that regulates neuronal differentiation and neuritogenesis (125). Overexpression of TTC3 in PC12 cells undergoing NGF-induced differentiation causes marked neurite retraction which is prevented by downregulation of CITK. Moreover, the inhibitory effects of TTC3 on PC12 differentiation require Rho activation but not ROCK (125). These data suggest that overexpression of TTC3, due to an extra copy of the DSCR on chromosome 21, could alter neuronal differentiation in the developing CNS through a Rho/CITK-dependent mechanism and contribute to cognitive impairment in DS.

6. ROLE OF RHO GTPASES IN NEURONAL DEATH AND DISEASE

6.1. Rho/ROCK and ischemia or excitotoxicity

In addition to their prominent roles in neuronal development and cognitive function, Rho family GTPases are critical players in neuronal death and disease. For example, both RhoA and RhoB have been implicated in the progression of neuronal death in ischemic injury or excitotoxicity, neuronal death provoked by over-excitation of glutamate receptors. In rat CGNs or in mouse cerebral cortex, RhoA is activated after either the addition of glutamate or occlusion of the middle cerebral artery, respectively (126). Excitotoxic stimulation of RhoA leads to the downstream activation of p38alpha MAPK, a stress-activated protein kinase, which ultimately induces apoptosis of CGNs. Similarly, a RhoA-p38alpha MAPK pro-apoptotic pathway is induced by NMDA in primary cortical and hippocampal neurons (126). In a murine stroke model, RhoB appears to be an early marker of neuronal death and may contribute to neurodegeneration by inducing profound actin cytoskeletal restructuring (127). Further evidence pointing to the involvement of RhoB in neuronal apoptosis after ischemia includes the observation that transgenic mice overexpressing X-chromosome linked inhibitor of apoptosis (XIAP) demonstrate markedly decreased RhoB expression and significantly reduced neuronal apoptosis following transient middle cerebral artery occlusion (128).

In addition to specific Rho isoforms being implicated in ischemic or excitotoxic neuronal death, ROCK, a downstream effector of Rho, also contributes to neuronal degeneration after these insults. In NMDA-induced excitotoxicity in the rat retina, both RhoA and ROCKII levels are significantly increased prior to neuronal death. Moreover, a ROCK inhibitor, fasudil, prevents the upregulation of both RhoA and ROCKII and reduces cell loss (129). In a similar manner, fasudil significantly protects against delayed neuronal death induced by ischemic injury in gerbils, even when administered as late as 24 hours after the ischemic insult (130). Finally, fasudil or another ROCK inhibitor, Y-27632, reduces cerebral infarct size and improves neurologic outcome in mice after middle cerebral artery occlusion (131). Collectively, the above findings demonstrate the potential utility of targeting the Rho/ROCK signaling pathway to minimize neuronal damage caused by stroke in humans (Figure 4).

6.2. Rho/ROCK and traumatic nerve injury

Rho/ROCK signals play a fundamental role in the inhibition of neurite outgrowth and therefore, activation of this pathway negatively influences the repair of CNS neurons after traumatic nerve injury (TNI) (132, 133). Upstream of the Rho signaling pathway, chondroitin sulfate proteoglycans (CSPGs) suppress axonal growth in scar tissue, and expression of inhibitory CSPGs is upregulated in lesioned spinal cord (134). Inhibition of either Rho with the Rho-specific inhibitor, C3 transferase, or ROCK with Y-27632, significantly blocks the inhibition of retinal axonal outgrowth by CSPGs (134). In addition, Rho GTPase is significantly activated following traumatic brain injury (TBI) in rats, though there is differential kinetics of Rho activation in various regions of the brain. For example, RhoA activation is seen as early as 24 hours post-injury and persists for up to three days post-injury in the cortex ipsilateral to the TBI, whereas RhoA activation is not observed in the hippocampus until three days post-injury (135). If the activation of RhoA is responsible for suppressing neurite outgrowth and impeding recovery after TNI, and is correlated with increased apoptosis, then interfering with the Rho GTPase signaling pathway may facilitate neuronal regeneration and survival after injury.

For example, in rat retinal ganglion cells (RGCs) subjected to microcrush lesion, regeneration of RGC axons is severely suppressed. In injured RGCs, however, the Rho inhibitor C3-07 enhances RGC axonal growth and prevents RGC death for up to one week after axotomy (136). Downstream of Rho GTPase, ROCK also plays a critical role in stifling axonal repair and functional recovery following TNI. For example, the ROCK inhibitor, Y27632, significantly enhances spinal cord axonal sprouting and stimulates recovery of several functional endpoints in rats subjected to cervical 4/5 spinal cord transection (137). Similarly, ROCK inhibition with Y27632 significantly reduces axon degeneration in transected cortico-spinal neurons (138). Finally, an examination of additional pathways that regulate ROCK activity revealed that the cell-cycle inhibitory protein p21 (CIP1/WAF1) is a ROCK-binding protein that suppresses its kinase activity. In rats that have undergone dorsal hemisection of the thoracic spinal cord, overexpression of a TAT-fusion protein of p21 promotes axonal regeneration and attenuates hindlimb dysfunction, further suggesting that prevention of ROCK activity may be beneficial in enhancing recovery after TNI (139). These studies demonstrate that the Rho/ROCK signaling pathway figures prominently in inhibiting axonal recovery from TNI, making this pathway a logical target to enhance neurite outgrowth and improve functional recovery following spinal cord injury and other forms of neurotrauma (Figure 4).

6.3. Rho/ROCK and Alzheimer's disease

In addition to ischemic injury and TNI, the pervasive neurological disorder, Alzheimer's disease (AD), may also progress, in part, through Rho family GTPase signaling. A recent study showed that both *in vitro* and *in vivo*, Rho and ROCK can promote increased levels of Abeta42 by regulating the processing of amyloid precursor protein (APP) (140). Abeta42 is an amyloidogenic peptide that underlies the pathogenesis of AD (141, 142). Furthermore, the increased levels of Abeta42 observed in a transgenic mouse model of AD could be reduced by a subset of nonsteroidal anti-inflammatory drugs (NSAIDs) that also demonstrate Rho inhibitory activity (140). Y-27632, a ROCK inhibitor that is protective in ischemia and spinal cord injury, also reduces levels of Abeta42 in AD transgenic mice, suggesting that Rho/ROCK inhibitors and specific Rho-inhibitory NSAIDs may be effective at slowing the progression of AD (140). In addition to Rho/ROCK regulation of APP processing and Abeta42 levels, Rac1 and Cdc42 GTPases may contribute to hippocampal neuronal death in AD by forming damaging F-actin aggregates downstream of Abeta42 signaling. In either hippocampal or cortical rat neurons stimulated with Abeta42, Rac1 and Cdc42 levels are upregulated and F-actin polymerization is enhanced. Moreover, the Abeta42-stimulated production of F-actin aggregates is inhibited by either dominant-negative Rac1 or Cdc42, showing that these GTPases control the accumulation of F-actin downstream of Abeta42 (143). These studies indicate that Rho family GTPases may play key roles in the deleterious production and signaling of Abeta42 that underlies AD pathology.

The above findings are in accordance with recent literature suggesting that chronic statin use is correlated with a reduced risk of AD. Statins inhibit the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase pathway, which forms mevalonate, a precursor for isoprenoid synthesis. Isoprenylation of Rho family GTPases is required to localize these proteins to the plasma membrane so that they can become activated (144, 145). Statins have recently been shown to block NADPH oxidase and subsequent reactive oxygen species generation in monocytes by inhibiting the isoprenylation of Rac1, thereby reducing inflammatory responses in the brain (146). These compounds similarly block the Abeta-induced expression of inducible nitric oxide synthase and nitric oxide production from microglia and monocytes (146). These anti-inflammatory actions of statins are likely due to an indirect inhibition of Rho family GTPase function via suppression of isoprenylation and membrane localization of the G-proteins (147). Finally, statins can alter APP processing to induce alpha-secretase-type shedding, producing an alpha-secretase cleaved soluble Alzheimer amyloid precursor protein (sAPPalpha) ectodomain (148). This type of APP processing inhibits the gamma-secretase-dependent formation of amyloidogenic Abeta peptide that forms toxic aggregates. The ability of statins to induce alpha-secretase shedding is blocked by a constitutively-active mutant of ROCK, while dominant-negative ROCK mimics the effects of statins, demonstrating that ROCK is a key regulator of APP processing. Additionally, active alpha-secretase can activate the nicotinamide adenine dinucleotide (NAD)+-dependent sirtuin-1 (SIRT1), a histone deacetylase that has been shown to reduce amyloid aggregates in a calorie-restriction model (149). However, ROCK inhibits the activation of SIRT1, suggesting another mechanism by which ROCK may enhance the accumulation of toxic amyloid aggregates in AD brain. The above findings indicate that suppression of Rho/ROCK signaling may be beneficial in decreasing amyloid load in AD (Figure 4).

6.4. Rho GTPases and polyglutamine diseases

Another group of neurological diseases that is characterized by aberrant aggregation of proteins include the polyglutamine diseases, such as spinal bulbar muscular atrophy (SBMA) and Huntington's disease (HD). Several lines of evidence suggest that Rho GTPases are involved in polyglutamine toxicity. Using a rapid cellular fluorescence resonance energy transfer (FRET) assay, Pollitt and colleagues showed that ROCK plays a central role in causing polyglutamine aggregation (150). The assay measured FRET between an overexpressed androgen receptor with an expanded tract of polyglutamine repeats (ARN127(65)CFP/YFP) and an overexpressed gluco-corticoid receptor with amino acids 108-317 removed (GRdelta), allowing the receptor to make aggregates more readily. Upon dexamethasone stimulation (to activate GRdelta), nuclear aggregates are formed and produce FRET. However, the ROCK inhibitor, Y-27632, effectively inhibits polyglutamine-induced FRET, results which are mimicked by the addition of a dominant-negative mutant of ROCK. Furthermore, Y-27632 blocks the aggregation of mutant huntingtin (htt) proteins transfected

into HEK293 cells. Finally, in a *Drosophila* model of polyglutamine neurodegenerative disease where photoreceptor neurons degenerate due to expression of htt with 93 added glutamines, Y-27632 reduces photoreceptor loss and protects from polyglutamine toxicity (150). The upstream signaling pathway that regulates ROCK in these models of polyglutamine toxicity is quite complex, however, and the specific Rho family member(s) involved has not been elucidated. Nevertheless, these data strongly implicate a role for Rho GTPases and ROCK in polyglutamine-induced neurodegeneration.

Recent data also suggest a role for Cdc42 signaling in HD. Utilizing a yeast two-hybrid screen, Holbert *et al.* (2003) revealed a role for Cdc42-interacting protein 4 (CIP4) in HD via a direct interaction with the htt protein (151). CIP4 is generally known for its role in modifying cytoskeletal architecture by binding to the Wiskott-Aldrich syndrome protein (WASP) and influencing actin and microtubule organization (152). *In vitro*, CIP4 interacts with the full-length form of htt (151). Moreover, in HD patients, CIP4 co-localizes with ubiquitin-positive aggregates and its expression increases proportionately with the severity of the disease. Finally, CIP4 overexpression in rat striatal neurons is sufficient to induce cell death, delineating a strong role for CIP4 in aggravating HD neuronal toxicity (151).

Other intermediary binding partners of the Rho GTPases may also significantly affect the progression of polyglutamine diseases. For example, insulin receptor substrate p53 (IRSp53) was found to mediate Rac-dependent recruitment of the WASP-related protein, WAVE, which stimulates actin polymerization at sites of membrane ruffling (153). This regulation occurs by Rac binding to IRSp53, uncovering its SH3 domain, which then recruits downstream effectors like WAVE to form a complex. IRSp53 similarly interacts with Dentatorubral-pallidoluysian atrophy (DRPLA) protein through the uncovered SH3 domain (154). DRPLA is a progressive neurodegenerative disease that results from an expanded polyglutamine tract in the gene and causes nuclear inclusions and aggregates in neurons. Though the DRPLA protein is expressed throughout the brain, only selective populations of neurons die in the disease, and IRSp53 mRNA appears to be localized to these areas (154, 155). Though there is no direct evidence of a Rac-IRSp53-DRPLA complex yet, Rac GTPase may allow IRSp53 to interact with DRPLA by uncovering its SH3 domain. These studies indicate a significant role for Rho GTPases in the formation of neurotoxic aggregates in polyglutamine diseases such as HD.

6.5. Rac/Cdc42 GTPases and sympathetic neuron death

Though Rho and ROCK are prominent players in neuronal damage and apoptosis in various disease models as shown above, Rac and Cdc42 are implicated exclusively in NGF withdrawal-induced apoptosis of rat sympathetic neurons isolated from the superior cervical ganglia (SCG). Bazenet and colleagues demonstrated that NGF withdrawal leads to a sequential activation of Cdc42 and Rac1 (156). Constitutively-active mutants of Rac and Cdc42 induce SCG neuron apoptosis in the presence of NGF, while dominant-negative mutants of these GTPases inhibit NGF withdrawal-induced death. In this model, stimulation of Cdc42 is responsible for increasing phosphorylation and transcriptional activity of the pro-apoptotic factor, c-Jun. Moreover, a transcriptionally-deficient mutant of c-Jun acts as a dominant-negative inhibitor of AP-1 activity and blocks Cdc42-induced neuronal apoptosis (156).

To determine the link between Cdc42 activity and c-Jun phosphorylation, the role of apoptosis signal-regulating kinase-1 (ASK1) was assessed. ASK1 is a MAPK kinase kinase, which activates both pro-apoptotic JNK and p38 MAPK pathways (157). Inactive mutants of ASK1 inhibit JNK activation and c-Jun phosphorylation, and protect SCG neurons from apoptosis induced by either NGF withdrawal or overexpression of constitutively-active Cdc42 (158). In addition, a dominant-negative mutant of c-Jun blocks cell death induced by a constitutively-active ASK1 mutant. These findings suggest that ASK1 can mediate signal transduction between Cdc42 and the pro-apoptotic JNK/c-Jun pathway.

Another mechanism by which Cdc42 can enhance c-Jun phosphorylation is through increasing the activity of mixed lineage kinase-3 (MLK3). MLK3 is a MAPK kinase kinase known to interact directly with Cdc42 through a CRIB motif to activate the JNK/c-Jun pathway (159). Overexpression of active MLK3 induces apoptosis of sympathetic neurons, while kinase dead (dominant-negative) mutant MLK3 significantly protects SCG neurons from apoptosis elicited by NGF withdrawal or overexpression of Cdc42, demonstrating a potential role for MLK3, in addition to ASK1, in linking Cdc42 activation to pro-apoptotic JNK/c-Jun signaling (160). The above studies reveal a pro-apoptotic signaling cascade via Cdc42 and Rac1-mediated activation of the JNK/c-Jun pathway, and contribute to the mechanistic understanding of how Rho GTPases can promote apoptosis of specific types of neurons.

7. ROLE OF RHO GTPASES IN NEURONAL SURVIVAL

7.1. Statin-induced neuronal death

In the above section of this review, we highlighted data indicating that chronic statin use is associated with a reduced risk of AD, possibly due in part, to the inhibitory effects of statins on Rho/ROCK-dependent APP processing and Abeta peptide formation (161). However, there is also considerable evidence that statins can be neurotoxic as a result of their inhibitory actions on Rho family GTPases. For example, more than a decade ago, Pavlov *et al.* (1995) reported that long-term exposure to lovastatin induces neuronal and glial cell death in fetal human brain explants (162). Similarly, various statins have been shown to induce apoptosis of either PC12 cells, rat brain neuroblasts, or primary rat cortical, hippocampal, or cerebellar granule neurons (163-167). Neurons exposed to statins *in vitro* show decreases in the synthesis of mevalonate and its metabolite, geranylgeranyl-pyrophosphate (GGPP), as well as diminished prenylation

and plasma membrane association of Rho family GTPases (164-167). Moreover, the plasma membrane localization of Rho GTPases is restored and neuronal apoptosis is prevented by co-incubation of statins with either mevalonate or GGPP (164, 165, 167). These results provide strong correlative evidence that statins induce neuronal apoptosis primarily by inhibiting the prenylation and membrane localization of Rho family members. Statin-induced neuronal apoptosis is associated with decreases in pro-survival Bcl-2 and Bcl-xL protein levels (164), induction of pro-apoptotic Bax (165), activation of the executioner caspase-3 (168, 169), and stimulation of JNK/c-Jun-dependent transcription of the pro-apoptotic, Bcl-2 homology-3 (BH3) domain-only protein, Bim (169). Bcl-2, Bcl-xL, Bax and Bim are all members of the Bcl-2 protein family which regulates intrinsic mitochondrial apoptosis (170). Thus, statins have the potential to induce neuronal death through inactivation of Rho family GTPases and consequent initiation of a mitochondrial apoptotic cascade that is modulated by Bcl-2 family proteins.

7.2. Rac and cerebellar granule neuron survival

Consistent with the data cited above for statin-induced neuronal apoptosis, we have found that primary rat CGNs are sensitive to apoptosis triggered by the specific inactivation of Rac GTPase. In our initial studies, we showed that CGNs undergo apoptosis when incubated with either *C. difficile* toxin B or *C. sordellii* lethal toxin, monoglucosyltransferases that display overlapping specificities for inhibiting Rac GTPase by direct glucosylation of a critical and highly conserved threonine residue (171, 172). In contrast, neither the specific inhibition of Rho with *C. botulinum* C3 ADP-ribosyltransferase, nor the direct disassembly of F-actin with *C. botulinum* C2 toxin, is sufficient to trigger CGN death (172). Furthermore, CGN apoptosis is similarly induced by adenoviral expression of dominant-negative Rac1, but not by dominant-negative mutants of RhoA or Cdc42 (173). CGN apoptosis induced by either toxin B, lethal toxin, or dominant-negative Rac1 is not prevented by serum or depolarizing potassium, suggesting that neither growth factors nor activity-dependent Ca^{2+} influx are able to compensate for the loss of Rac function. Moreover, CGN apoptosis elicited by inhibition of Rac is dependent on activation of the pro-apoptotic transcription factor, c-Jun, which occurs upstream of the induction of Bim, stimulation of Bax-dependent cytochrome c release, and intrinsic apoptosis (173). Finally, we have shown that Rac1 is itself a caspase substrate during CGN apoptosis and that the inactivation and cleavage of Rac leads to the rapid downregulation of a PAK/MEK/ERK1/2 signaling cascade that normally promotes degradation of pro-apoptotic Bim (174). These results are similar to the apoptosis pathway elicited by statins in that both Rac-inactivating toxins and dominant-negative Rac ultimately induce neuronal death by triggering a mitochondrial apoptotic cascade. In agreement with our findings, PAK activity has also been demonstrated to play a key role in the preservation of CGNs cultured in depolarizing concentrations of extracellular potassium. Overexpression of dominant-negative PAK induces apoptosis of CGNs in depolarizing medium, while constitutively-active PAK rescues CGNs from apoptosis induced by removal of the depolarization stimulus (175). Rac/PAK signaling may promote CGN survival by actively suppressing the induction of Bim and consequent initiation of Bax-dependent mitochondrial apoptosis, suggesting that cultured CGNs depend on Rac GTPase function for their survival *in vitro*.

The above findings suggest that Rac signaling should also be vital for the survival of CGNs *in vivo*. To date, the precise role of Rac in preserving CGN survival *in vivo* has not been investigated, although Rac1 is clearly expressed in these neurons in intact cerebellum (176). An indication of the potential importance of Rac GTPase signaling in CGN survival *in vivo* can be gleaned from studies on the weaver mutant mouse model of cerebellar ataxia. These mice display a mutation in the inwardly rectifying potassium channel, GIRK2, which leads to CGN death soon after birth and consequent development of severe ataxia (177). Previous work has demonstrated that the Rac-specific GEF, Tiam1, is highly expressed in CGNs of wild-type mice but is absent from pre-migratory CGNs of weaver mutant mice at a stage immediately prior to detectable cell death (22). Although it is presently unclear by what mechanism Tiam1 expression is downregulated in weaver mouse CGNs, it is interesting to note that these neurons die by apoptosis and furthermore, Tiam1 is known to be susceptible to caspase-mediated degradation (178-180). Moreover, CGNs identified as undergoing apoptosis in weaver mutant mice by terminal dUTP nick-end labeling (TUNEL) assay show co-staining for active c-Jun and demonstrate increased expression of Bax (179, 181). Though indirect, these data suggest that loss of Tiam1 function, perhaps via caspase cleavage, may lead to Rac inactivation and subsequent stimulation of c-Jun- and Bax-dependent CGN apoptosis in the weaver mutant mouse. This pro-apoptotic pathway is analogous to that induced in cultured CGNs by the inhibition of Rac. In future studies, it will be interesting to determine if the weaver mutant mouse also shows Bim induction in dying CGNs and if Bim deletion is neuroprotective in this disease model. Further investigation is still necessary to definitively establish if Rac function is indeed crucial for CGN survival *in vivo*.

7.3. Rho GTPases and motor neuron survival

CGNs are not the only neuronal population suggested to show dependency on Rho family GTPase function for their survival during development. Using a conditional Cre-*loxP* expression system driven by the dopamine beta-hydroxylase gene promoter, Kobayashi *et al.* (2004) demonstrated that mice transgenic for dominant-negative mutants of either RhoA or ROCK display enhanced apoptotic death of developing spinal motor neurons during early embryogenesis (182). Similarly, in primary cultures of embryonic rat spinal motor neurons, expression of a dominant-negative mutant of Rac1 induces marked cell death and causes suppression of axon outgrowth in the remaining motor neurons (183). These two studies indicate that spinal motor neurons, like CGNs, likely have a requirement for Rho and/or Rac function for their survival both *in vitro* and *in vivo*.

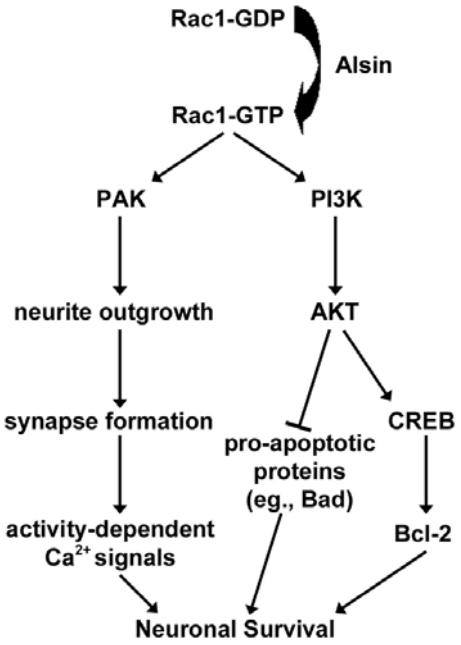

Figure 5. Rac-dependent pro-survival pathways activated downstream of alsin in neurons. The Rac1-GEF, alsin, induces GTP-loading of Rac1 which leads to the activation of PAK and PI3K. PAK signaling stimulates neurite outgrowth, synapse formation, and activity-dependent Ca^{2+} signals, all of which promote neuronal survival. PI3K induces activation of AKT which promotes survival by phosphorylating and inhibiting several pro-apoptotic proteins. AKT can also stimulate the transcriptional activity of CREB to induce expression of the anti-apoptotic Bcl-2 protein. CREB, cAMP response element-binding protein; PAK, p21-activated kinase; PI3K, phosphatidylinositol 3-kinase.

7.4. Loss of Rac function in amyotrophic lateral sclerosis

Additional evidence of an essential role for Rac function in the normal development and survival of spinal motor neurons comes from the recent identification of the causative gene mutated in a recessive form of juvenile-onset amyotrophic lateral sclerosis (ALS), ALS2, a degenerative disorder involving substantial death of spinal motor neurons (183). The ALS2 gene encodes a unique GEF, alsin, which demonstrates dual specificity for Rac1 and Rab5 GTPases (184-186). To date, alsin mutations have been detected in at least three distinct infantile- or juvenile-onset degenerative disorders of motor function including ALS2, familial juvenile primary lateral sclerosis, and infantile-onset ascending hereditary spastic paralysis (184, 187-189). In all of these cases, observed mutations in the ALS2 gene result in a truncated alsin protein which is predicted to be deficient in GEF function. The alsin protein is widely expressed in the developing CNS including neurons of the cerebral cortex, brain stem, spinal cord, and cerebellum, with the highest levels of expression peaking during early postnatal life, a critical period in the activity-dependent differentiation and development of spinal motor neurons (190, 191).

The GEF activity of alsin towards Rab5 is through its vacuolar protein sorting 9 (Vps9) domain (186, 192). Through its Rab5 GEF function, alsin appears to modulate early endosome fusion and trafficking. For example, primary neurons isolated from ALS2 null mice show deficiencies in the endosomal trafficking of various neurotrophin receptors, suggesting that ALS2 mutations which cause loss of alsin function may result in impaired neurotrophin signaling (193). Interestingly, the Rab5-dependent function of alsin appears to be regulated by Rac since alsin interacts directly with active Rac1 (194). GTP-bound Rac1 apparently recruits alsin to sites of membrane ruffling and macropinosome formation, the latter of which alsin converts to early endosomes via Rab5-dependent fusion. Thus, the GEF activity of alsin towards Rab5 is actually regulated by Rac1 function and therefore, alsin can be considered a downstream effector of Rac (194).

The GEF activity of alsin for Rac1 occurs via its Dbl homology domain (186), and this Rac1 GEF function appears to play a critical role in the pro-survival effects of alsin in motor neurons. For example, alsin co-localizes with Rac in neuronal growth cones and stimulates Rac-dependent PAK activity and neurite outgrowth (195). Neuritogenesis is required for proper synapse formation during development which ultimately promotes the activity-dependent survival of motor neurons (191). In addition, delivery of small interfering RNA against alsin via electroporation knocks down expression of the alsin protein and induces significant axonal degeneration and cell death in cultured embryonic rat spinal motor neurons (183). These deleterious effects of alsin depletion are prevented by expression of a constitutively-active mutant of Rac1 but are unaffected by constitutively-active Rab5 (183). These data suggest that the ability of alsin to promote spinal motor neuron survival, at least *in vitro*, is strictly dependent on its Rac1 GEF activity and is unrelated to its Rab5 GEF function. The mechanism by which the Rac1 GEF activity of alsin promotes motor neuron survival is presently unclear; however, several hypotheses have been put forward. For instance, deletion analysis of alsin revealed that its ability to protect motor neurons from mutant Cu/Zn superoxide dismutase (SOD1)-induced neurotoxicity, the cause of some autosomal dominant forms of ALS, is dependent on presence of the Rac1 GEF domain (196). Moreover, alsin interacts directly with mutant SOD1, but not with wild-type SOD1, via the Rac1 GEF domain, suggesting that alsin may protect motor neurons via sequestration of toxic proteins. Besides a direct protein:protein interaction effect, alsin also activates a specific anti-apoptotic signaling cascade in NSC34 motor neuronal cells to further mitigate mutant SOD1-induced toxicity. Overexpression of alsin protects NSC34 cells from mutant SOD1 and this effect is lost in cells depleted of Rac1 (197). Downstream of Rac1, both phosphatidylinositol 3-kinase (PI3K) and an isoform of AKT are required for alsin-dependent neuroprotection against mutant SOD1. Finally, *in vitro* studies in the SK-N-BE neuronal cell line have demonstrated that overexpression of alsin exerts a neuroprotective action by increasing the ratio of anti-apoptotic Bcl-xL to pro-apoptotic Bax protein levels (198). Conversely, a mutant

form of alsin associated with familial juvenile primary lateral sclerosis decreases this protein ratio and induces neuronal apoptosis. Interestingly, Rac is known to stimulate a number of transcription factors (*eg.*, cAMP response element-binding protein, CREB) which are capable of regulating the expression of Bcl-2 family proteins (199-201). Collectively, these studies indicate that alsin promotes motor neuron survival via multiple Rac-dependent signaling pathways (Figure 5). Thus, loss-of-function mutations in alsin which are causative in several degenerative motor neuron diseases likely induce motor neuron death principally by disrupting Rac GTPase function.

8. SUMMARY AND PERSPECTIVE

Since the discovery by Hall and colleagues in the early 1990s that Rho GTPases are central regulators of actin cytoskeletal dynamics, an expansive amount of work has been published on this protein family. A major observation emanating from this large body of research is that Rho family GTPases function in diverse facets of cell biology. Indeed, their effects on actin are a key element in the regulation of neuronal growth cone dynamics and dendritic spine morphogenesis, which ultimately contribute to normal synaptic plasticity and proper cognitive development. However, the novel role of Rho family GTPases in modulating neuronal survival and death is rapidly emerging as a fascinating area for new exploration, particularly in the realm of clinical development for neurotrauma and neurodegenerative diseases.

On the one hand, Rho GTPases and their effectors can exert deleterious effects on neuronal function and survival. For example, Rho/ROCK signaling appears to play a central role in enhancing injury after ischemic or excitotoxic insults, impairing recovery following TNI, and increasing amyloid load in AD (Figure 4). In the context of drug development for these disorders, one might consider novel Rho or ROCK inhibitors to have promising therapeutic potential. This is in fact the case for TNI, where the recombinant protein Rho antagonist, Cethrin, is currently undergoing clinical evaluation for acute spinal cord injury (202). Similarly, the inhibitory effects of statins on Rho/ROCK signaling, though indirect, likely contribute to their potential to reduce the risk of developing AD (203). Finally, as a means of mitigating neuronal damage due to stroke, Rho/ROCK inhibitors might be an option; though recent findings suggest that p38 MAPK, which is activated downstream of Rho/ROCK in ischemia and excitotoxicity, may also be a candidate drug target (126). Clearly, elucidation of the detrimental actions of Rho family GTPases in various neuronal disorders has expanded the possible therapeutic avenues for neurotrauma and some forms of neurodegeneration.

In contrast to the above findings, Rho GTPases can also demonstrate essential pro-survival effects in specific types of neurons. For example, CGNs which can degenerate in certain forms of ataxia, appear to depend on the activity of Rac GTPase for their survival (172-174). Similarly, Rac GTPase function is essential for the survival of spinal motor neurons (183). This is perhaps best evidenced by the recent discovery that alsin, the product of the ALS2 gene which is mutated in several forms of juvenile-onset motor degeneration, promotes motor neuron survival principally through its Rac1 GEF activity (Figure 5). Given that alsin mutations are the underlying cause of ALS2, this debilitating neurodegenerative disease can essentially be considered a disorder caused by the loss of Rac1 function. Therefore, defining the pro-survival signaling pathways activated in neurons downstream of Rac is critical for the identification of novel strategies to combat this disease. Moreover, discovery of pro-apoptotic pathways induced in neurons by the specific loss of Rac GTPase function may also provide novel therapeutic targets to slow or inhibit neuronal death in disorders such as ALS2.

Undoubtedly, as research continues in the area of Rho GTPase signaling in the nervous system, a better understanding of effector pathways, as well as GEF and GAP specificities for particular Rho family members, will be attained. Given the crucial roles that these G-proteins play in neuronal development and survival, further illumination of the signaling pathways regulated by Rho family GTPases in specific neuronal populations is a major goal of future research. As the diverse roles of Rho family GTPases in neuronal survival and death continue to be unraveled, novel therapeutic targets for neurodegenerative disorders involving this protein family will be revealed.

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Abbreviations: alphaPIX: alpha-p21-activated kinase interacting exchange factor; ACK: activated Cdc42-associated kinase; AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; APP: amyloid precursor protein; ASK1: apoptosis signal-regulating kinase-1; BH3: Bcl-2 homology-3; CGN: cerebellar granule neuron; CIP4: Cdc42-interacting protein 4; CITK: citron kinase; CNS: central nervous system; CREB: cAMP response element-binding protein; CRIB: Cdc42/Rac-interactive binding; CRMP-2: collapsin response mediator protein-2; CSPG:

chondroitin sulfate proteoglycan; DRPLA: Dentatorubral-pallidoluysian atrophy; DS: Down syndrome; DSCR: DS Critical Chromosomal Region; ERM: ezrin-radixin-moesin; ERK1/2: extracellular signal-regulated kinases 1 and 2; FMRP: fragile X mental retardation protein; FRET: fluorescence resonance energy transfer; GAP: GTPase-activating protein; GEF: guanine nucleotide exchange factor; GGPP: geranylgeranyl-pyrophosphate; GPCR: G-protein-coupled receptor; HD: Huntington's disease; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzymeA; htt: huntingtin; IRSp53: insulin receptor substrate p53; JNK: c-Jun-NH2-terminal kinase; LIMK: LIM kinase; MAPK: mitogen-activated protein kinase; MLCK: myosin light chain kinase; MLK3: mixed lineage kinase-3; MRX: nonspecific X-linked mental retardation; NAD+: nicotinamide adenine dinucleotide; NGF: nerve growth factor; NMDA: N-methyl-D-aspartate; NSAID: nonsteroidal anti-inflammatory drug; PAK: p21-activated kinase; PI3K: phosphatidylinositol 3-kinase; RGC: retinal ganglion cell; ROCK: Rho kinase; SBMA: spinal bulbar muscular atrophy; sAPPalpha: alpha-secretase cleaved soluble Alzheimer amyloid precursor protein; SCG: superior cervical ganglia; SIRT1: sirtuin-1; SH3: Src-homology-3 domain; SOD1: Cu/Zn superoxide dismutase; TBI: traumatic brain injury; TNI: traumatic nerve injury; TSC: tuberous sclerosis complex; TTC3: tetratricopeptide repeat domain 3; TUNEL: terminal dUTP nick-end labeling; Vps9: vacuolar protein sorting 9; WASP: Wiskott-Aldrich syndrome protein; XIAP: X-chromosome linked inhibitor of apoptosis

Key Words: Rho, Rho kinase, Rac, p21-activated kinase, Alsin, Neuronal apoptosis, Neuronal survival, Review

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